Remarks

Claims 38-45, 64-71, 90-97, and 116-123 have been cancelled herein without prejudice or disclaimer. Applicants reserve the right to pursue the subject matter encompassed by all cancelled claims in one or more divisional or continuation applications. Claims 37 and 115 have been amended to correct redundant claim dependencies and to correspond to the claim dependencies as found in these claims when originally submitted. *See*, Provisional Election With Traverse, pages 4 and 13, submitted October 9, 2001. Upon entry of the present amendments claims 21-24, 26-37, 46-49, 51-63, 72-76, 78-89, 98-101, 103-115, and 124 will be pending.

Newly Asserted Rejections -

Claim Rejections under 35 U.S.C. § 112, first paragraph

Claims 73-76, 78-87, and 98-101 were rejected under 35 U.S.C. § 112, first paragraph. *See*, Paper No. 27, page 2, item 4. In particular, it was asserted that "a suitable deposit for patent purposes is suggested." It was also asserted that "Applicant has not disclosed the deposit of the cell line, ATCC-97129, nor does the specification provide any nexus between ATCC-97129 and SEQ ID NO:2." *See*, Paper No. 27, page 3, first and second paragraphs.

Applicants respectfully disagree and traverse. Applicants submit that a suitable deposit for patent purposes was submitted before the earliest benefit date in the present application. This is evidenced by the terms of the specification as filed, for example, at page 5, last paragraph the specification teaches:

> In accordance with an aspect of the present invention, there are provided isolated nucleic acids (polynucleotides) which encode for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the cDNA of the clone(s) deposited as ATCC Deposit No. 97129 on April 28, 1995.

Hence, the specification teaches that a cDNA clone (*i.e.*, nucleic acids/polynucleotides) encoding polypeptides of the invention was deposited on April 28, 1995. Furthermore, in view of the teachings in the present specification, it would also be understood by those of ordinary skill in

the art that the polynucleotide encoding SEQ ID NO:2 and the cDNA clone in ATCC Deposit No. 97129 represent to the same gene. *See also, for example*: specification page 6, second and third paragraphs; page 7, second through fourth full paragraphs; and, page 10, first full paragraph. Accordingly, the present specification provides a nexus between ATCC Deposit No. 97129 and SEQ ID NO:2.

The Examiner has requested the filing of an affidavit or declaration "[i]f a deposit has been made under the provisions of the Budapest Treaty..." to provide assurances regarding availability of the deposit. *See*, Paper No. 27, page 3, third paragraph to page 4, first paragraph. Additionally, it was also asserted that "Applicant's provision of these assurances would obviate this objection/rejection." *See*, Paper No. 27, page 4, first full sentence. In compliance with this request, Applicants herein provide the following statement, attested to by the signature of the Agent for the Applicants below:

Availability of the Deposit

> Human Genome Sciences, Inc., the assignee of the present application, has deposited biological material under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209 (present address). The deposit was made on April 28, 1995, accepted by the ATCC, and given ATCC Accession Number 97129. In accordance with M.P.E.P. § 2410.01 and 37 C.F.R. § 1.808, assurance is hereby given that all restrictions on the availability to the public of ATCC Accession Number 97129will be irrevocably removed upon the grant of a patent based on the instant application, except as permitted under 37 C.F.R. § 1.808(b). A partially redacted copy of the ATCC Deposit Receipt for Accession Number 97129 is enclosed herewith.

The Examiner also requested amendment of the specification "to recite the date of deposit and the complete name and address of the depository." *See*, Paper No. 27, page 4, third paragraph. To comply with this request Applicants have herein amended the paragraph at page 6, line 1, which was inserted before the first paragraph by preliminary amendment, to replace "ATCC" with "American Type Culture Collection (ATCC)." Accordingly, Applicants submit

that the specification as amended herein and as amended in the initial preliminary amendment, recites the date of deposit and the complete name and address of the depository.

In view of the above amendment, explanation, and affirmation, Applicants respectfully request that the rejection of claims 73-76, 78-87, and 98-101 under 35 U.S.C. § 112, first paragraph, be withdrawn.

Claim Rejections under 35 U.S.C. § 112, second paragraph

Claims 29, 55, and 81 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly "being indefinite" due to recitation of the term "chimeric" antibody. *See*, Paper No. 27, page 4, last two paragraphs. In particular it was stated that:

> The term is not defined by the specification and the exact meaning of the word chimeric is not known. The term chimeric is generic to a class of antibodies which are products of genetic shuffling of antibody domains and other active proteins. The term encompasses antibodies fused to non-immunoglobulin proteins as well as antibodies wherein any domain of the antibody is substituted by corresponding regions or residues of human antibodies including but not limited to CDR grafted antibodies.

See, Paper No. 27, page 4, last paragraph to page 5, first paragraph.

Applicants respectfully disagree with and traverse the rejection. In particular, Applicants disagree with the assertion that "the exact meaning of the word chimeric is not known." As an initial matter, Applicants note that the Examiner has properly characterized the term chimeric antibodies as "generic to a class of antibodies..." and as encompassing "antibodies fused to non-immunoglobulin proteins as well as antibodies..." Moreover, Applicants submit that the term "chimeric" antibodies was routinely used and understood by those of ordinary skill in the art as of the earliest claimed benefit date in the present application. In evidence of this assertion, Applicants submit herewith copies of three publications describing chimeric antibodies. *See*, Baier, *et al.*, "Immunogenic Targeting of Recombinant Peptide Vaccines to Human Antigen-Presenting Cells by Chimeric Anti-HLA-DR and Anti-Surface Immunoglobulin D Antibody Fab Fragments In Vitro", *Jour. Virol.*, v.69, n.4, pp.2357-2365 (April 1995); Poon, *et al.*, "Structure and Function of Several Anti-Dansyl Chimeric Antibodies Formed by Domain Interchanges between Human IgM and Mouse IgG2b", Jour. Biol. Chem., v.270, n.15, pp.8571-8577 (April 1995); and, Maloney, *et al.*, "Phase I Clinical Trial Using Escalating Single-Dose Infusion of

Chimeric Anti-CD20 Monoclonal Antibody (IDEC-C2B8) in Patients With Recurrent B-Cell Lymphoma", *Blood*, v.84, n.8, pp.2457-2466 (October 1994). Applicants note that these three publications represent a small sampling of the numerous publications on chimeric antibodies which were available on or before the earliest claimed benefit date in the present application.

Applicants also disagree with that portion of the rejection which asserted that "The term [chimeric] encompasses antibodies fused to...antibodies wherein any domain of the antibody is substituted by corresponding regions or residues of human antibodies..." *See*, Paper No. 27, page 5, first full sentence (emphasis added). Applicants point out that the pending claims do not allow for "any domain" to be substituted in the claimed antibodies because the claimed chimeras are limited to antibodies that specifically bind polypeptides of the invention. Hence, antibody binding domains of the claimed chimeric antibodies cannot be substituted for domains which do not specifically bind polypeptides of the present invention. In other words, the claimed chimeric antibodies are limited to antibodies which retain domains that specifically bind the Colon Specific Protein of the present invention.

Accordingly, in view of the above evidence and explanations, Applicants submit that the term "chimeric" antibodies is not indefinite. It is respectfully requested that the rejection of claims 29, 55, and 81 under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.

Claim Rejections under 35 U.S.C. § 102

Claims 21-24, 26, 28, 35, 46-49, 51-52, 54, 61, 73-76, 78, 80, 87, 98-101, 103-104, 106, 113, and 124 were rejected under 35 U.S.C. § 102(a) and (e) as allegedly anticipated by U.S. Patent No. 5,436,169. *See*, Paper No. 27, page 5, item 7. In particular, it was asserted that:

> US Patent No. 5,426,169[1] teaches a polypeptide, SEQ ID NO:7 with 7 amino acids identical to those of SEQ ID NO:2, flanked by a single conservatively substituted amino acid and teaches SEQ ID NO:12, the polynucleotide encoding said polypeptide...since the encoded protein comprises 7 amino acids, identical to SEQ ID NO:2, which are flanked by a conservatively substituted amino acid, it would be expected that a subset of the polyclonal antibodies that specifically bind said encoded protein would bind also to SEQ ID NO:2, given it is understood by those of

[1] In view of the Examiner's initial reference to U.S. Patent No. 5,436,169 and to "us-09-525-041-2.rni, Result 11" Applicants assume the Examiner intended to cite U.S. Patent No. 5,436,169 (instead of 5,426,169); if this assumption is incorrect, clarification is requested.

> ordinary skill in the art that the minimum epitope for antibody binding is 5-6 amino acids and that antibodies will bind to epitopes with conservative amino acid substitutions.

See, Paper No. 27, page 6, last paragraph.

Applicants respectfully disagree and traverse. U.S. Patent No. 5,436,169 (hereinafter the "'169 patent") teaches that "the cDNA of the human PAP [pancreatitis-associated protein] is characterized in that it codes for the protein corresponding to the following A3 amino acid sequence (SEQ ID NO:7)." *See*, U.S. Patent No. 5,436,169, column 6, lines 33-36. The human PAP sequence of SEQ ID NO:7 shares approximately 30% identity with the Colon Specific Protein of the present invention, with the longest contiguous stretch comprising 7 amino acids. *See enclosed,* Sequence Alignment Colon Specific Protein vs. U.S. Patent No. 5,436,169 SEQ ID NO:7. Further, the '169 patent teaches:

> Human PAP proteins according to the invention are also characterized in that their amino acid sequences exhibit a homology of at least 50%, and preferably at least 60% and in the most preferred case at least 70% of at least one sequence of about 25 amino acids included in the A2 sequence of the mature PAP protein of the rat, or in the A3 sequence of the human PAP protein described above.

See, '169 patent, column 8 lines 28-47. Thus, the '169 patent does not contemplate polypeptides with only 30% identity as part of the invention disclosed therein. Furthermore, the presently pending claims are drawn to antibodies that specifically bind Colon Specific Protein of the present invention. Thus, the presently pending claims encompass antibodies that bind human Colon Specific Protein and species orthologs[2] (including fragments of at least 50 contiguous amino acids) but not paralogs[2] or other homologous proteins such as the human PAP protein described in the '169 patent. In other words, antibodies that cross-react with human PAP of the '169 patent and Colon Specific Protein of the present invention are not encompassed by the

[2] The terms "ortholog/orthologue" and "paralog/paralogue" are used herein as these terms are generally understood and in accord with a definition provided by the National Center for Biotechnology (NCBI). *See* the NCBI Field Glossary available on the internet at http://www.ncbi.nlm.nih.gov/Class/FieldGuide/glossary.html#A, a printout of the excerpted definitions for orthologue and paralogues is included herewith. Orthologous genes (or, genes which are orthologues) are derived from a common ancestor through vertical evolutionary descent. Thus, genes (and proteins encoded by them) are considered to be orthologues when they represent the *same* gene (or protein) found in *different* species. For example, feline FGF-1 (fibroblast growth factor-1), murine FGF-1, and human FGF-1 are orthologues (orthologous proteins). In contrast, paralogous genes (or, genes which are paralogues) are genes found within the same genome that are thought to have evolved by gene duplication. For example, human FGF-1, human FGF-2, and human FGF-3 are gene paralogues; and, the proteins encoded by these genes are also paralogues (or paralogous proteins).

presently pending claims. Therefore, the claimed antibodies of the present invention are not anticipated by U.S. Patent No. 5,436,169.

Applicants additionally note, the fact that an antibody specifically binds a polypeptide in one species (*e.g.*, human) and also binds the orthologous polypeptide in another species (*e.g.*, mouse) does not change characterization of that antibody as "specific" by those of ordinary skill in the art. Thus, an antibody that specifically binds human Protein-X can also specifically bind murine Protein-X. In contrast, an antibody that binds equally well to human Protein-X and human Protein-Y (*i.e.*, paralogous proteins) would not be an antibody that "specifically" binds Protein-X. Consider, for example, the 1994 *Boehringer Mannheim Biochemicals Catalog* which used the term "specifically" to describe an antibody that bound both murine and human Mac-1 protein. In particular, the *Boehringer Catalog* listed an Anti-Mac-1 (macrophage associated antigen) (clone M1/70) antibody and described it under the heading "Specificity and Notes":

> The antibody specifically reacts with native mouse and human Mac-1 (complement receptor type 3; Ly-40) antigen and precipitates two chains, 170kD (CD11b) and 95kD (CD18).

See, Boehringer Mannheim Biochemicals, Inc. 1994 Catalog, page 260, included herewith (emphasis added). This is just one example of an antibody described as "specifically" binding orthologous proteins in different species.

In view of the above explanation, Applicants respectfully request that the rejection of claims 21-24, 26, 28, 35, 46-49, 51-52, 54, 61, 73-76, 78, 80, 87, 98-101, 103-104, 106, 113, and 124 under 35 U.S.C. § 102(a) and (e) be reconsidered and withdrawn.

Claim Rejections under 35 U.S.C. § 103

Claims 21-24, 26, 28, 35, 46-49, 51-52, 54, 61, 73-76, 78, 80, 87, 98-101, 103-104, 106, 113, and 124 were rejected under 35 U.S.C. § 103 as allegedly being "unpatentable over Bartoli et al (FEBS Letters, 1993, 327:289-293, also see us-09-525-041-2.rsp, [R]esult 6, attached) in view of US Patent No. 5,436,169." *See*, Paper No. 27, page 8, item 9.

Applicants respectfully disagree and traverse. In particular, since U.S. Patent No. 5,436,169 does not comprise an anticipatory reference for the reasons provided above, this reference may not properly be combined with *Bartoli et al.* to allege obviousness of the presently pending claims. Furthermore, as explained above, the presently pending claims are drawn to

antibodies that specifically bind Colon Specific Protein and species orthologs (including fragments of at least 50 contiguous amino acids). Therefore, despite the presence "eight homologous/conservatively substituted amino acids", the presently pending claims do not encompass antibodies that cross-react with protein paralogs such as Reg and Reg1 (discussed in *Bartoli et al)*. *See*, Paper No. 27, page 98, fourth paragraph.

In view of the above explanation, Applicants respectfully request that the rejection of claims 21-24, 26, 28, 35, 46-49, 51-52, 54, 61, 73-76, 78, 80, 87, 98-101, 103-104, 106, 113, and 124 under 35 U.S.C. § 103 be reconsidered and withdrawn.

Rejoinder of Method Claims

The Examiner has refused to rejoin method claims with the presently pending product claims. *See*, Paper No. 27, page 10, item 10. In order to expedite prosecution of the present application, method claims 38-45, 64-70, 90-97, 116-123 have been cancelled herein. Applicants reserve the right to pursue the subject matter encompassed by all cancelled claims in one or more divisional or continuation applications.

Objection to Allowable Claims

Claims 27, 30-34, 36-37, 53, 56-60, 63-63, 72, 89, 105, 107-112, 114-115 were "objected to as dependent upon rejected claims, but would be allowable if rewritten in independent form..." *See*, Paper No. 27, page 10, item 12.

Applicants respectfully disagree and traverse the above objection. As an initial matter, claim 72 is currently written in independent form, therefore no amendment of this claim should be required. Additionally, Applicants submit that in view of the evidence and explanations provided herein, the independent claims upon which the dependent claims rely should be allowed. Thus, it is respectfully submitted that each of the currently pending claims are independent, or depend from an allowable independent claim.

Finally, Applicants note that the claims drawn to monoclonal antibodies have been designated as encompassing allowable subject matter, whereas the claims drawn to polyclonal antibodies have been rejected. The rejection of polyclonal antibody claims appears to be based on the allegation that U.S. Patent No. 5,436,169 is an anticipatory reference. In particular, in rejecting polyclonal antibody claims based on the '169 patent it was asserted that:

> Since the encoded protein [in U.S. 5,436,169] comprises 7 amino acids, identical to SEQ ID NO:2 [of the present specification], which are flanked by a conservatively substituted amino acid, it would be expected that a subset of the polyclonal antibodies that specifically bind to said encoded protein would bind also to SEQ ID NO:2, given that it is understood by those of ordinary skill in the art that the minimum epitope for antibody binding is 5-6 amino acids and that antibodies will bind to epitopes with conservative amino acid substitutions.

See, Paper No. 27, page 6, last paragraph (emphasis added). Applicants respectfully submit, however, that such explanation does not provide a logical legal distinction for allowing claims drawn to monoclonal antibodies but denying claims drawn to polyclonal antibodies. For example, it could just as logically be said that "it would be expected that a subset of the monoclonal antibodies that specifically bind to said encoded protein would bind also to SEQ ID NO:2." However, as explained above, the presently pending claims, whether drawn to monoclonal or polyclonal antibodies, do not encompass antibodies that cross-react with PAP of U.S. Patent No. 5,436,169 or antibodies that cross-react with Reg or Reg1 of *Bartoli, et al.* Therefore, claims drawn to both monoclonal and polyclonal antibodies should be allowable.

In view of the above explanations, it is respectfully requested that the objection to claims 27, 30-34, 36-37, 53, 56-60, 63, 72, 89, 105, 107-112, and 114-115 be reconsidered and withdrawn.

Conclusion

Should the Examiner have any questions regarding the present reply, Applicants would welcome a personal interview with the Examiner prior to further substantive examination.

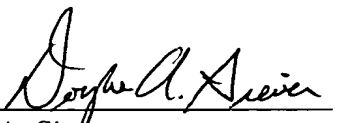
Applicants respectfully request that the above-made amendments and remarks be entered and made of record in the file history of the instant application. The Examiner is invited to call the undersigned at the phone number provided below if any other further action by Applicant would expedite the examination of this application.

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37

C.F.R. § 1.136, such an extension is requested and the fee should also be charged to our Deposit Account.

Dated: April 7, 2004

Respectfully submitted,

By 

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KKH/DAS/mr

American *Type Culture* Collection

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Human Genome Sciences, Inc.
Attn: Robert H. Benson, Ph.D.
9410 Key West Avenue
Rockville, MD 20850

Deposited on Behalf of: Human Genome Sciences, Inc.

Identification Reference by Depositor:	**ATCC Designation**
DNA Plasmid, 376867 (PF178)	97129

The deposits were accompanied by: _ a scientific description _ a proposed taxonomic description indicated above.

The deposits were received April 28, 1995 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested May 4, 1995. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Date: May 2, 1995

Annette L. Bade, Director, Patent Depository

cc: Greg D. Ferraro, Esq.

RP

JOURNAL OF VIROLOGY, Apr. 1995, p. 2357–2365
0022-538X/95/$04.00+0
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Vol. 69, No. 4

Immunogenic Targeting of Recombinant Peptide Vaccines to Human Antigen-Presenting Cells by Chimeric Anti-HLA-DR and Anti-Surface Immunoglobulin D Antibody Fab Fragments In Vitro

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Received 19 October 1994/Accepted 15 January 1995

To increase the inherently weak immunogenicity of synthetic peptide vaccines, we used recombinant DNA techniques to generate chimeras between immunogenic determinants of human immunodeficiency virus type 1 (HIV-1) gp120 and antibody Fab fragments reactive with surface structures displayed specifically on human antigen-presenting cells (APCs), including surface immunoglobulin D (sIgD) and class II major histocompatibility complex (MHC) molecules. Hybridomas producing anti-human MHC class II (HLA-DR) or surface immunoglobulin D monoclonal antibodies (MAbs) that recognize nonpolymorphic determinants were used to clone chimeric Fab gene fragments by employing an established procedure to generate antigen-binding Fab libraries in phagemid vector pComb3. Molecular and immunochemical analysis indicated that the expected chimeric Fab fragments expressing the HIV-1 epitopes were correctly cloned and expressed in *Escherichia coli* and retained the binding specificity of the native (hybridoma-derived) MAb. The chimeric Fab fragments targeted the linked HIV-1-derived antigenic determinants to the surface of human APCs in vitro, as evidenced by fluorescence-activated cell sorter analysis. Furthermore, such recombinant immunotargeted HIV-1 peptide antigens demonstrated improved immunogenicity over equivalent nonimmunotargeted control antigens, as shown by their ability to stimulate interleukin-2 production by $CD4^+$ T-helper cells from human donors exposed to HIV-1 antigens. These data suggest that immunotargeting of recombinant peptide antigens via the attached Fab fragments facilitates uptake by human APCs with subsequent access to the MHC class II processing pathway, thereby validating the immunotargeting concept for such recombinant subunit vaccines in an in vitro human system.

Synthetic peptides constitute one major strategy for the development of a new generation of vaccines against infectious diseases, including AIDS (3–5). Peptides encompassing pathogen-derived T-plus-B-cell epitopes can function as complete immunogens that elicit humoral and cell-mediated immunity (17, 45, 52, 58). The potential advantages offered by such subunit vaccines include easy preparation of a pure immunogen in large quantities, safety, cost effectiveness, stability, and easy storage and delivery. In addition, by selecting defined epitopes that stimulate desirable protective immunity, it may be possible to eliminate pathogen-derived protein sequences that elicit undesirable responses, including general toxicity, immunosuppression, enhancement of antibodies, or autoimmunity, properties ascribed to certain retroviral (including human immunodeficiency virus type 1 [HIV-1]) antigens (8, 11, 12, 34).

One major obstacle to the effective use of synthetic peptide vaccines in humans is inherently weak immunogenicity. Strategies that potentiate the immune response to peptide immunogens are likely to greatly improve the practical value of synthetic peptides as vaccines (4, 17, 52, 58). Since the efficiency of antigen presentation to T cells depends, to a large extent, on the concentration of antigen-major histocompatibility complex (MHC) complexes on the surfaces of antigen-presenting cells (APCs) (29), it can be anticipated that any technique that increases the concentration of immunogenic peptides displayed by APCs will lead to more effective antigen presentation and, hence, successful vaccination. Approaches that have been used to achieve immunotargeting, as evidenced by increased antigen-specific antibody or T-helper (Th) cell responses, include the use of homogeneous antigen-specific B-cell populations as APCs (40, 49) and polyclonal antigen targeting to mouse B cells by using rabbit anti-mouse immunoglobulin (Ig) as an antigen (20, 56).

Another immunotargeting approach involves covalent coupling of protein or synthetic peptide antigens to antibodies specific for determinants displayed on APCs, e.g., surface Ig (sIg) on B cells or anti-MHC class II antibodies on B cells and other APCs such as macrophages or dendritic cells. The feasibility of this approach was established with rodent models by several independent studies. Covalent coupling of protein or short peptide antigens to anti-Ig or anti-MHC antibodies enhanced the specific immune response by 10^1- to 10^3-fold at the level of both antigen-specific T-cell proliferation in vitro (15, 16, 38, 50) and antibody responses in vivo (13, 14, 38, 43). Such antigens were sometimes found to be highly immunogenic in the absence of oil-based adjuvants (13, 14). sIg cross-linking by the antibody was not essential, since monovalent Fab fragments also amplify the immune response, albeit with ~10-fold lower efficiency (15, 16). Several other surface molecules, e.g., class I MHC (16, 50) or a dendritic cell-specific antigen (43), but not CD11/18 (14), CD45, or FcγRII (50), can also serve as targets for antigen focusing.

With covalent coupling, it is nearly impossible to control the antigen-to-immunotargeting antibody ratio or coupling site,

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TABLE 1. Oligonucleotide primers and probes used in this study[a]

Designation	Sequence
cDNA consensus primer	
C_{H2} (γ1, γ2a, γ2b, γ3)	5'-ACCAC(C/A)ACACA(T/C)GTGA-3'
Heavy-chain PCR primers:	
VRP_H	5'-AGGT(C/G)(C/A)A(G/A)CT(G/T)CTCGAGTC(T/A)GG-3'
CRP_{H1} (γ1)	5'-TATGCAACTAGTAGATCTACAATCCCTGGGCACAATTTTCTT-3'
CRP_{H1} (γ2a, γ2b, γ3)	5'-ACAGGGACTAGTAGATCTGGGCACTCTGGGCTCAATTTTCTT-3'
Light-chain PCR primers:	
VRP_κ	5'-CCAG(A/T)T(G/C)(T/C)GAGCTC(C/G)(A/T)(C/G)(C/A)T(C/G)AC(C/A)CAG(A/T)CTCCA-3'
V3-CRP_κ	5'-GCGCCGTCTAGAATTATTTTCCTATTGTAACAAATGCTCTCCCTGGTCCTCTCTGGATACG GGATCCACACTCATTCCTGTTGAAGCTCTTGAC-3'
ANP-C_κ	5'-GCGCCGTCTAGAATTATTTTCCTATTGTAACAAA-3'
Internal oligonucleotide hybridization probes:	
C_κ-IP	5'-ACTGCCATCAATCTTCCACTTGAC-3'
C_{H1}-IP	5'-GAA(A/G)TA(G/A)CCCTTGACCAGGCATCC(C/T)AG-3'

[a] CRP, constant-region primer; VRP, variable-region primer; ANP, anchor primer. Underlined nucleotides represent restriction enzyme sites used for cloning.

thus complicating the task of optimizing vaccine design with no (or minimal) batch-to-batch variability. To overcome this difficulty, we constructed chimeric genes that encode fusion proteins of antibody fragments expressing short, immunogenic HIV-1 peptides. Construction of such chimeras at the gene level allows coupling of the desired combination of epitopes to the proper antibodies under precise conditions, including choice of coupling sites and epitope-to-antibody ratio. The availability of convenient restriction sites also allows the preparation of multivalent subunit vaccines by replacement or addition of oligonucleotide sequences that specify defined epitope combinations, e.g., a cocktail of variable pathogen sequences which often coincide with immunodominant epitopes with relative ease, enabling the construction of potential multivalent vaccines.

To determine whether the immunotargeting approach, generally studied only with rodents, can be extended to the human system, chimeric anti-human HLA-DR or sIgD antibodies incorporating an immunodominant V3 loop peptide, P18, and a potent Th epitope, EnvT1 (T1), both derived from the gp120 envelope glycoprotein of HIV-1_{IIIB}, were expressed in *Escherichia coli* and purified as monovalent Fab fragments. These chimeric Fabs bound specifically to human APCs displaying the relevant HLA-DR or sIgD molecules and demonstrated improved immunogenicity as measured by increased stimulation of interleukin-2 (IL-2) production in vitro by human $CD4^+$ Th cells from donors exposed to HIV-1 antigens.

MATERIALS AND METHODS

Cell lines and reagents. Murine hybridomas L203 (IgGγ1, Lκ; ATCC HB1710), BP107.2.2 (IgGγ3, Lκ; ATCC TIB154), and IA6-2 (IgGγ2a, Lκ) were used in this study. Human IgD δ chain and HIV-1_{IIIB} gp120 were purchased from Chemicon Int., Temecula, Calif., and ABT, Cambridge, Mass., respectively. Purified HLA-DR protein was a kind gift from H. Grey, Cytel Corp., La Jolla, Calif.

RNA isolation and cDNA synthesis. Total RNA from hybridoma cell lines was isolated by the RNAzol B method (Cinna/Biotecx, Friendswood, Tex.). In general, 10^6 cells were lysed in 100 μl of RNazol B solution; this was followed by phenol-chloroform extraction and isopropanol precipitation in accordance with the manufacturer's protocol. Two micrograms of total RNA was used as the template for oligo(dT) (Lκ cloning)- or C_{H2} (Fd cloning)-primed first-strand cDNA synthesis with Superscript RNase H^- reverse transcriptase (Gibco-BRL, Gaithersburg, Md.) by the standard method (see Table 1 for the C_{H2} cDNA primer used).

DNA amplification with consensus Ig PCR primers. Fab-encoding cDNAs were amplified in standard PCRs. The various PCR primers and oligonucleotide probes used are listed in Table 1. The PCR mixture included ~0.5 μg of the cDNA template, 0.3 μg of primers, and deoxynucleoside triphosphate 200 μM each in *Taq* polymerase buffer containing 1.5 mM $MgCl_2$ and 5 U of *Taq* polymerase (Promega, Madison, Wis.) in a final volume of 100 μl. PCRs were conducted with a TwinBlock thermal cycler (Ericomp, San Diego, Calif.). The primers used for amplification of mouse heavy-chain sequences (Fd) were VRP_H and the appropriate subclass-specific CRP_{H1}, and the reactions were performed for 30 cycles under the following conditions: 91°C for 1 min, 52°C for 2 min, 72°C for 1 min, and a final elongation for 15 min at 72°C. To amplify and clone chimeric Lκ fragments encoding the HIV-1 V3 loop peptide (P18) in a single step, we designed hybrid primers containing Lκ-plus-P18-specific nucleotide sequences. The primers used for amplification of mouse chimeric light-chain sequences (Lκ) were VRPκ (5') and a mixture of 3' primers consisting of hybrid primer V3-CRPκ and the corresponding primer ANP-Cκ at a molar ratio of 9:1. Temperature cycles were as follows: 5 cycles at 91°C for 1 min, 45°C for 2 min, and 72°C for 1 min; 25 cycles at 91°C for 1 min, 55°C for 2 min, and 72°C for 1 min; and a final elongation step for 15 min at 72°C. The identities of these PCR-amplified fragments were confirmed by Southern hybridization with internal oligonucleotide probes Cκ-IP and C_{H1}-IP (Table 1).

Construction of phage display libraries. Surface expression libraries were constructed in two steps. First, amplified Fd fragments were digested at 37°C for 12 h with an excess of restriction enzymes *Xho*I and *Spe*I and size fractionated by agarose gel electrophoresis. DNA fragments were purified and directionally ligated into *Xho*I-*Spe*I-linearized and phosphatase-treated vector pComb3. Following transformation of *E. coli* XL1-Blue, 100 ml of 2× TB medium (6) containing ampicillin (100 μg/ml) was added and cultures were grown overnight at 37°C. Phagemid DNA containing the Fd fragment library was isolated, linearized with *Sac*I-*Spe*I, and phosphatase treated, and the recombinant 4.7-kb vector fragment was purified by agarose gel electrophoresis. Next, amplified chimeric Lκ fragments were digested with an excess of restriction enzymes *Sac*I and *Xba*I at 37°C for 12 h and size fractionated by agarose gel electrophoresis. The Lκ cDNA fragments were then cloned into the Fd-containing, *Sac*I-*Spe*I-linearized, phosphatase-treated vector pComb3. Following transformation of *E. coli* XL1-Blue, recombinant phage were generated as previously described (6).

Panning of the combinatorial library to select antigen binders. Microtiter plates were coated with 1 μg of purified HLA-DR or IgD protein overnight at 4°C. The wells were blocked, incubated with the phage display libraries, washed, and eluted with 100 mM glycine buffer (pH 2.5). The eluted phage were then used to infect *E. coli* XL1-Blue cultures. New phage stocks were prepared and reincubated with antigen in microtiter wells as described above. After three consecutive rounds of panning, bacterial colonies expressing chimeric Fabs with the desired antigen specificity were detected in a filter assay in which colonies were grown for 16 h at 37°C on Durapore master filters in close contact with a second (Immobilon P) membrane coated with anti-mouse Ig antibodies. This was done on top of Luria broth agar dishes containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 μg of ampicillin per ml. Secreted bacterium-derived Fabs diffusing onto the second membrane were thereby immobilized by the capture antibodies and detected by specific binding to a radiolabeled antigen (31). By this procedure, colonies expressing antibody fragments with the desired specificity were easily identified and isolated for further analysis.

Preparation of soluble Fab fragments containing three HIV-1 peptides. Phagemid DNA from positive clones was isolated, and the *Bgl*II-*Nhe*I fragment encoding phage coat protein pIII was replaced with artificial gene fragments encoding two tandem HIV-1 peptides (P18 and T1) plus a flexible linker se-

quence (Gly-Gly-Gly-Gly-Ser) and convenient flanking restriction sites. The tandem peptide sequence was synthesized as slightly overlapping single-stranded oligonucleotides, kinase treated, annealed, and ligated with *Bgl*II-*Nhe*I-linearized Fab phagemid DNA. The second strand was filled in with the Klenow fragment of DNA polymerase I before bacterial transformation by standard procedures. Plasmid DNAs from positive clones were sequenced by the dideoxy method with Sequenase 2.0 (U.S. Biochemical Corp.) and analyzed with Mac Vector sequence analysis software.

Expression and purification of chimeric Fabs. Bacterial clones were grown at 26°C in 2× TB medium containing 100 μg of ampicillin per ml and 20 mM $MgCl_2$ until an optical density at 600 nm of 0.8 was achieved. IPTG (0.1 mM) was added, and the culture was induced for 24 h at 26°C. Pelleted (4,000 rpm, 30 min, Sorvall GSA rotor) *E. coli* cells were resuspended in phosphate-buffered saline (PBS) containing 2 mM phenylmethylsulfonyl fluoride and lysed by sonication on ice. Following removal of debris by centrifugation (14,000 rpm, 30 min, Sorvall SS-34 rotor), the filtered supernatant was affinity purified by overnight batch absorption at 4°C with a Sepharose-coupled anti-mouse Lκ antibody (Zymed, San Francisco, Calif.). After extensive washing with PBS, chimeric Fabs were eluted with acidified PBS-HCl (pH 2.5), neutralized with 1 M Tris base (pH 9.0), and concentrated by Centriprep-30 (Amicon, Beverly, Mass.) ultrafiltration. Endotoxin contamination was removed by using Detoxi-Gel (Pierce, Rockford, Ill.) as described by the manufacturer. The Fab preparations were dialyzed against PBS, concentrated to ~1 mg/ml, and stored at 4°C. The concentrations of chimeric Fabs were determined by measuring A_{280} with an extinction coefficient of 1.56, and purity was analyzed by nonreducing sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and Coomassie blue R-250 staining.

Western blot (immunoblot) analysis. Purified chimeric Fab fragments were separated by nonreducing SDS–10% PAGE and analyzed by immunoblotting with polyclonal rabbit anti-mouse IgG antibodies or with biotin-labeled H902, an anti-gp120 (HIV-1_{IIIB}) monoclonal antibody (MAb) specific for P18 (19) and then a goat anti-rabbit IgG- or streptavidin-peroxidase conjugate, respectively, and a chemiluminescence detection kit (ECL reagent; Amersham Corp., Arlington Heights, Ill.).

IL-2 production. Healthy volunteers aged 18 to 45 years and HIV-1-seropositive, asymptomatic, nonanergic homosexual males (with CD4 counts between 400 and 760 and reactivity to the purified protein derivative test immunogen) were recruited, and their peripheral blood mononuclear cells (PBMC) were purified by centrifugation over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.). PBMC (2×10^5) were stimulated in 7-day triplicate cultures supplemented with 5% normal, heat-inactivated human AB serum and 2 μg of anti-IL-2 receptor MAb Tac per ml (to prevent consumption of secreted IL-2 by the activated cells), and IL-2 titers in triplicate culture supernatants were determined with CTLL cells as previously described (21, 22, 24, 25, 48). Data are expressed in terms of a stimulation index, which was calculated by dividing the mean counts per minute incorporated in antigen-containing cultures by the mean counts per minute in cultures without antigen (medium control). The datum points shown are for a culture supernatant dilution of 1:4.

RESULTS

Choice of model epitopes and cloning of chimeric Fab. The epitope chosen for initial analysis was P18 (NH_2-RIQRG PGRAFVTIGK-COOH), a 15-residue peptide corresponding to amino acids 315 to 329 in the V3 loop region of HIV-1_{IIIB}-derived envelope glycoprotein gp120. This well-characterized antigen was chosen because it represents the principal neutralization domain (37, 41), as well as an epitope recognized by MHC-restricted cytotoxic T lymphocytes (CTL) (8, 54, 55). The MHC class I-restricted nature of the CTL response to this epitope (54), its high variability among HIV-1 isolates (41), and the lack of sufficient human Th activity provided by it (42, 45) prompted us to clone and express chimeric Fabs displaying, in addition to P18, the T1 peptide, a well-defined Th epitope (18) represented by the sequence NH_2-KQIINMWQEVGKA MYA-COOH (amino acids 428 to 443 in $gp120_{IIIB}$). T1 was found to be immunogenic in the context of several human MHC haplotypes (9, 21, 22, 24, 25, 48) and to provide help for neutralizing antibody (45) or CTL (42) responses specific for the covalently linked V3 loop epitope.

The targeting anti-human MAbs selected were L203 and IA6-2, which recognize monomorphic determinants of the HLA-DR and sIgD molecules expressed by all human haplotypes, respectively. BP107.2.2, an anti-mouse MHC class II MAb which does not bind to human APCs, was used as a control. To facilitate the cloning and expression of multiple antigen-binding Fab fragments, we made use of the well-established procedure for assembling combinatorial, antigen-binding Fab libraries on phage surfaces by using modified phagemid vector pComb3 (6). To amplify and clone chimeric Lκ fragments encoding the HIV-1-derived P18 sequence in a single step, we designed hybrid primers containing Lκ- and P18 peptide-specific nucleotide sequences. A panning procedure was used to select and enrich antigen-binding phage clones by adsorbing the phage display libraries on microtiter plates coated with purified antigens, i.e., human HLA-DR or IgD. After three consecutive rounds of panning, bacterial colonies expressing chimeric Fabs with the desired antigen specificity were detected in a filter assay by specific binding to a radiolabeled antigen (data not shown). To prepare soluble Fabs containing additional HIV-1 peptide sequences, vector pComb3 was modified by replacing the M13 phage coat pIII sequence with an artificial gene fragment that encodes the T1 and P18 peptides in tandem. The resulting pComb3-chimeric Fab expression system, containing two copies of P18 and one copy of T1, is shown schematically in Fig. 1.

Expression and characterization of chimeric Fabs. Chimeric Fabs were expressed in *E. coli* as monovalent Fab fragments to test their biological activities in vitro. Methods used to express and purify them in sufficient quantities were optimized and resulted in production levels of ~50 to 300 μg of chimeric Fab per liter of bacterial culture. Yields were improved by isolating the antibody fragments from the periplasmic space rather than from culture supernatants (data not shown). Therefore, cell lysates were used directly for affinity purification of chimeric Fabs. Purified material was analyzed by SDS-PAGE and Coomassie R-250 staining (Fig. 2A) or immunoblotting with polyclonal rabbit anti-mouse IgG (Fig. 2B) or MAb H902, a V3 loop-specific anti-$gp120_{IIIB}$ MAb (Fig. 2C). Both antibodies reacted specifically with ~58-kDa proteins, confirming the expression of chimeric, HIV-1 peptide-expressing Fab fragments.

Antigen binding and expression of P18 by the chimeric Fab were independently demonstrated in an enzyme-linked immunosorbent assay in which microtiter plates were coated with the appropriate antigen, e.g., purified HLA-DR or IgD molecules, and then incubated with the chimeric Fabs. Fab binding was detected by using anti-P18 MAb H902 as a probe. Recombinant L203 and IA6-2 retained the binding specificity of the native (hybridoma-derived) MAb (Fig. 3), thereby confirming the functional integrity of these antibody Fab fragments. No binding was detected with recombinant chimeric control Fab BP107.2.2 or with L203 and IA6-2 and their nonappropriate antigens. The specificity of chimeric Fab binding to the immobilized antigens, or that of H902 binding to the P18-expressing chimeric Fab, was further indicated by the ability to block both binding reactions by preincubation with their respective ligands, i.e., excess purified HLA-DR or IgD, or free P18, respectively (Fig. 3, groups 4 and 5).

The binding of chimeric Fabs to HLA-DR or sIgD molecules displayed on the surfaces of appropriate target cells was evaluated by immunofluorescence and fluorescence-activated cell sorter analysis with fluorescein isothiocyanate-labeled $F(ab')_2$ anti-mouse Ig antibodies as secondary reagents. Recombinant chimeric Fab fragments retained binding activity comparable to that of native, hybridoma-derived, intact antibodies L203 and IA6-2 or its Fab fragments (data not shown). Moreover, focusing of P18 on the surfaces of human peripheral blood lymphocytes (PBL) by the chimeric L203 or IA6-2 Fab, but not by control Fab BP107.2.2, was verified by immunofluorescent staining with the P18-specific MAb (Fig. 4A). The small fraction of PBL stained by chimeric IA6-2 is compatible with the proportion of $sIgD^+$ B cells in blood (35). As before,

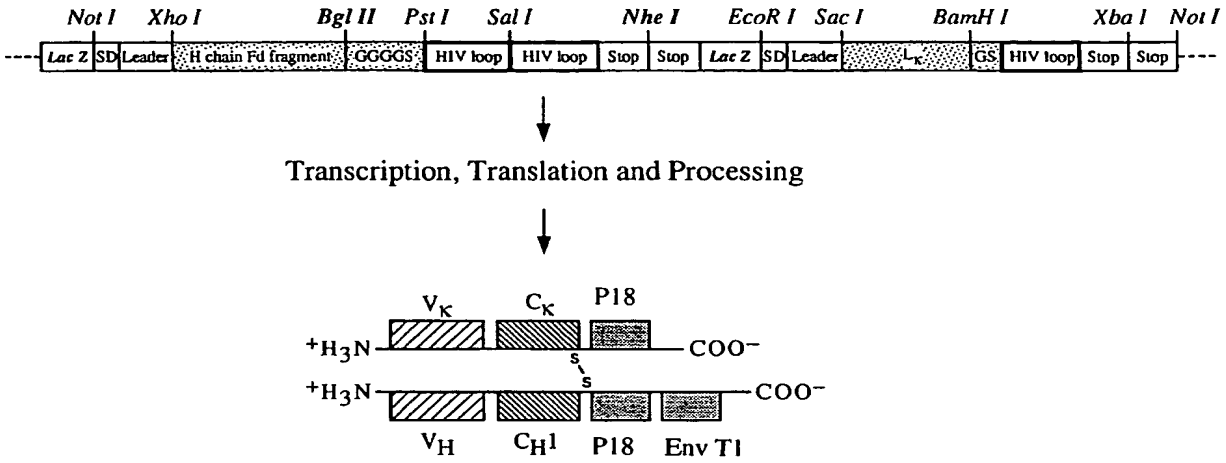

FIG. 1. Structure of the recombinant pComb3 vector system used for expression of chimeric Fab fragments in *E. coli* XL-1 Blue and schematic representation of the chimeric HIV-1 epitope-presenting Fabs. SD, Shine-Dalgarno sequence.

binding was blocked by preincubation of the chimeric Fabs with excess purified HLA-DR or IgD or by pretreating the secondary H902 antibody with free P18 (data not shown). Furthermore, neither L203 nor IA6-2 reacted with murine or rat APCs.

The anti-human HLA-DR chimeric L203 Fab also reacted with spleen cells from a *Cynomolgus* monkey (Fig. 4B). Since L203 recognizes a monomorphic human HLA-DR epitope, this reactivity most likely represents conservation of this epitope between humans and other primates. L203 immunoreactivity (which was blocked by preincubation with excess soluble human HLA-DR) was also observed with baboon lymphoblastoid cell line 26CB-1 (ATCC CRL1495). This interspecies immunological cross-reactivity may eventually allow testing of L203-immunotargeted recombinant peptide vaccines with primate models of HIV infection.

Stimulation of human T cells from HIV-1-exposed donors. To assess the in vitro immunogenicity of the T1- and P18-expressing chimeric Fabs, we used them as antigens to stimulate PBMC from randomly selected HIV-positive, asymptomatic, nonanergic donors or from HIV-negative, age-matched, healthy volunteers. Binding of the L203 and IA6-2 MAbs to monomorphic MHC class II or IgD determinants, respectively, allowed us to use these immunogens with PBMC of different donors irrespective of their MHC (and/or IgD) polymorphisms. In addition to chimeric L203 and IA6-2 and control (nonbinding) BP107.2.2, other, related antigens were used for comparison. These included the free P18 and T1 peptides and a recombinant HIV-1_{IIIB} gp120 protein, as well as tetanus toxoid (TT), which was used as a recall antigen (21, 22, 24, 25, 48). T-cell activation by these antigens was assessed by measuring IL-2 titers in antigen-stimulated and unstimulated culture supernatants. This assay was shown to be considerably more sensitive than proliferation assays based on [^{3}H]thymidine incorporation for detection of virus-specific Th activity among HIV-positive donors (24).

Since only limited amounts of blood from HIV-positive, nonanergic donors were available, preliminary titration experiments were conducted to optimize the conditions for detection of an immunotargeting effect (data not shown). These experiments utilized PBMC from one HIV-seropositive donor who responded well to the corresponding synthetic HIV-1 peptides. Titration of the chimeric Fabs over a range of 25 to 400 μg/ml indicated that maximal differences in stimulation by immunotargeted versus nonimmunotargeted antigens were obtained at a chimeric Fab concentration of 25 μg/ml. This antigen concentration, which is equivalent, on a molar basis, to 1.4 or 0.75 μg of the free P18 or T1 peptide, respectively, per ml, was used in subsequent experiments.

The HIV-1-seropositive Walter Reed stage 1 patients studied were only those whose PBMC generated greater-than-twofold IL-2 responses when stimulated with both TT and the synthetic HIV-1 peptides. PBMC from six of eight HIV-1-positive donors (and all HIV-1-negative donor-derived

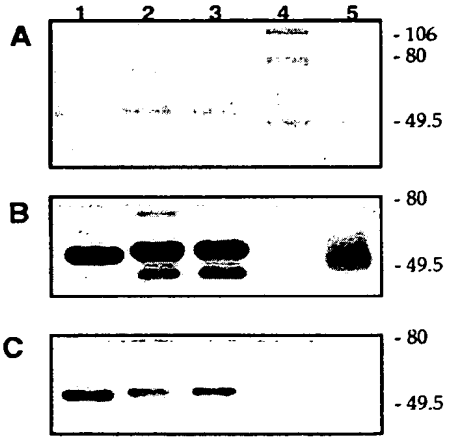

FIG. 2. Coomassie blue R-250 staining (A) and immunoblot analysis of purified chimeric Fabs with polyclonal rabbit anti-mouse IgG antibodies (B) or anti-gp120 MAb H902, which is specific for V3 loop peptide P18 (C). Both antibodies reacted specifically with the expressed Fab fragments, indicating that the corresponding HIV-1 epitope is also correctly expressed. Bands of ~58 kDa represent assembled Fab heterodimers of chimeric L203 (lane 1), IA6-2 (lane 2), or BP107.2.2 (lane 3). The positions of molecular size standards (lane 4) and the native ~50-kDa Fab fragment prepared by papain cleavage of a purified hybridoma-derived L203 MAb (lane 5) are shown. The numbers on the right are molecular sizes in kilodaltons.

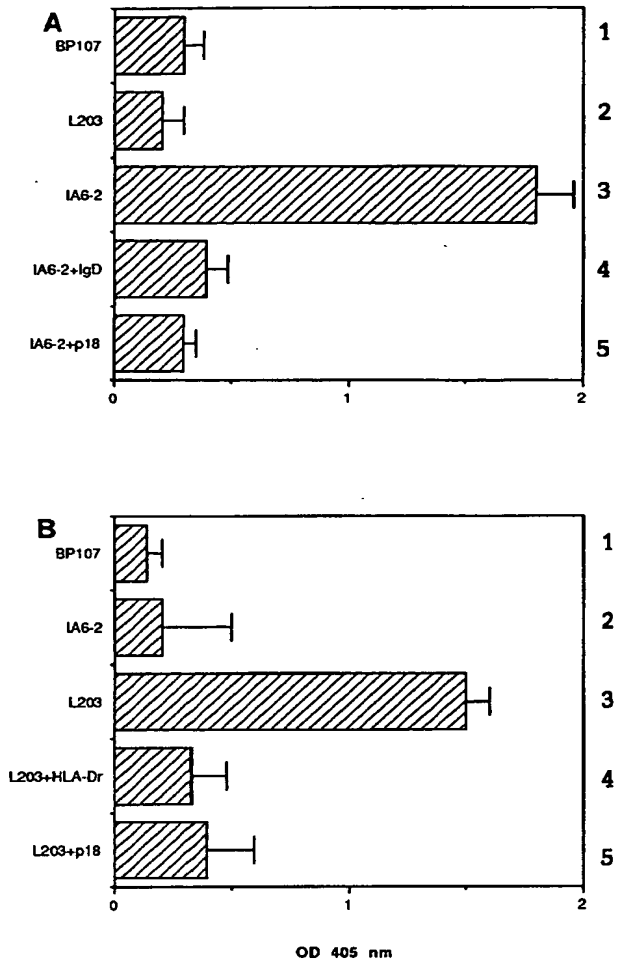

FIG. 3. Enzyme-linked immunosorbent assay analysis of the binding specificity of purified chimeric Fabs. Binding anti-human HLA-DR (L203) and sIgD (IA6-2) and nonbinding anti-mouse MHC class II (BP107.2.2) Fabs were added to wells coated with either purified human sIgD (A) or HLA-DR (B) molecules. In some cases, the chimeric Fab was preincubated in the presence of excess soluble antigen (group 4) or peptide P18 (group 5). Binding was detected by biotinylated anti-P18 MAb H902, followed by an avidin-alkaline phosphatase conjugate and the corresponding color reaction. Chimeric L203 or IA6-2 did not bind to other protein antigens, e.g., mouse I-A^d or IgD, bovine serum albumin, or lysozyme (data not shown). OD, optical density.

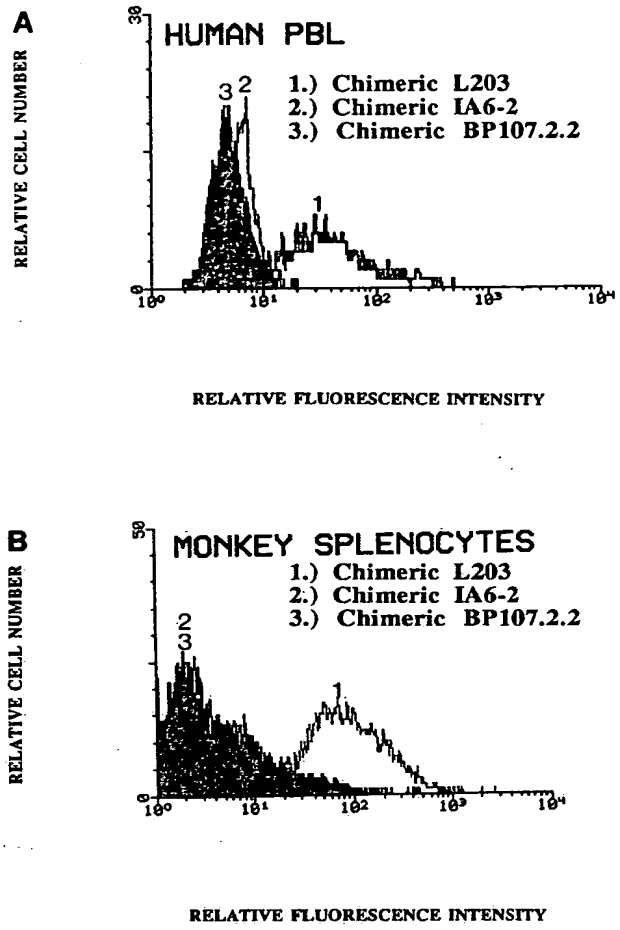

FIG. 4. Focusing of HIV-1-derived loop epitope P18 on the surfaces of human lymphocytes (A) or cryopreserved (>90% viable) *Cynomolgus* monkey spleen cells (B) by chimeric anti-human HLA-DR (L203) or anti-human sIgD (IA6-2) Fabs. Cells were stained with a purified Fab, i.e., L203 or IA6-2 (or nonbinding BP107.2.2 as a negative control), and then exposed to biotinylated H902 and fluorescein isothiocyanate-labeled streptavidin. Positive staining indicates focusing of the HIV-1 epitope to the cells. As shown, this particular anti-human HLA-DR Fab (L203) also binds to primate APCs. Staining with an intact hybridoma-derived MAb and then exposure to fluorescein isothiocyanate-$F(ab')_2$ rabbit anti-mouse IgG produced identical results (data not shown). PBL, peripheral blood lymphocytes.

PBMC) responded to the recall antigen, and PBMC from four of them also responded to the HIV-1_{IIIB} peptide antigens. The IL-2 responses of these four donors, as well as those of two representative healthy controls, are shown in Fig. 5. Whereas all six donors responded to TT, only PBMC from HIV-1-positive donors responded with significant IL-2 production to stimulation with the HIV-1-derived antigens, indicating the antigen-specific nature of the Th response. The two chimeric Fabs that were capable of targeting the attached P18 and T1 epitopes to the surfaces of APCs, i.e., L203 and IA6-2, stimulated a significantly stronger response than did an equivalent concentration of nonbinding chimeric Fab BP107.2.2 or recombinant $gp120_{IIIB}$ (each at 25 μg/ml). Similar IL-2 production induced by free P18 or T1 required high peptide concentrations (~150 μg/ml) that represent a 100- to 200-fold excess over the concentration of the same peptides in the context of the chimeric Fabs. At concentrations equimolar to those of the chimeric Fabs, the free peptides failed to induce detectable IL-2 production (Fig. 6).

Next, we addressed the possibility that the increased immunogenicity of the targeting chimeric Fabs is due to polyclonal activation of antigen-presenting B, or other, cells. Thus, the immunogenicity of the chimeric Fabs was compared with that of a mixture of the corresponding native (nonchimeric) Fabs, prepared by papain cleavage with the free synthetic peptides. In addition, the synthetic peptides alone were tested as immunogens, either at a low concentration (1.5 μg of each per ml as a mixture of P18 and T1) equivalent to their concentration in

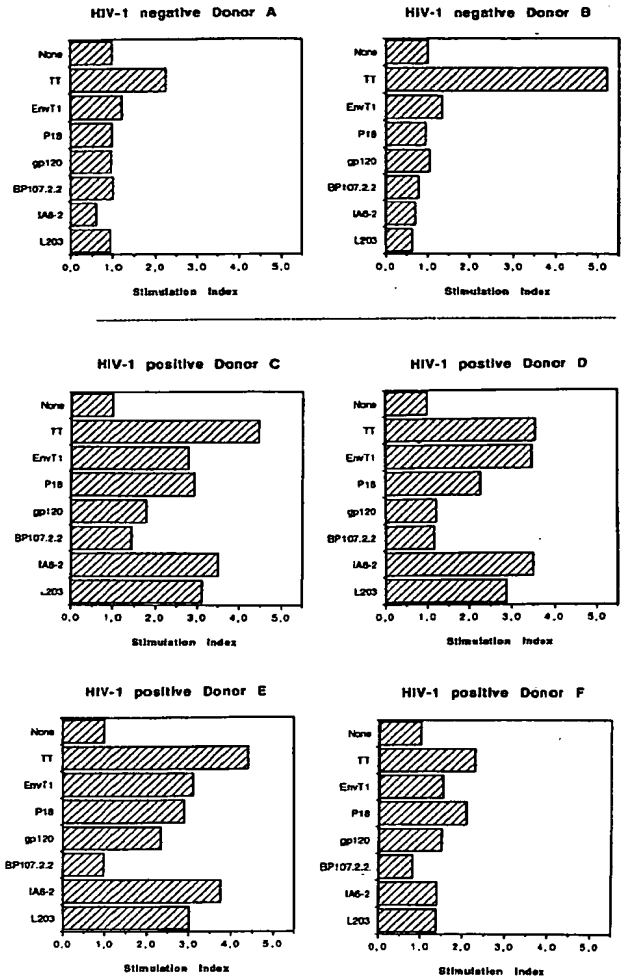

FIG. 5. Enhanced IL-2 production of human T cells by immunotargeted recombinant peptide vaccines. PBMC (2×10^5) were cultured for 7 days in the absence or presence of the antigens indicated. Antigen concentrations were as follows: TT, $gp120_{IIIB}$, or chimeric Fab, 25 μg/ml; free synthetic peptide P18 or T1, 150 μg/ml. IL-2 production was assayed by the ability of culture supernatants to support the growth of CTLL cells as measured by [^{3}H]thymidine incorporation. Data are expressed as stimulation indexes. Responses of HIV-1-seronegative donors A and B to TT were 16,848 and 16,668 cpm, and those of the corresponding medium controls were 7,448 and 3,200 cpm, respectively. Responses of PBMC from the four HIV-1-positive donors in the absence (medium control) and presence of TT (in counts per minute) were as follows: donor C, 2,008 and 8,936; donor D, 1,800 and 6,372; donor E, 1,140 and 5,032; donor F, 1,832 and 4,216, respectively.

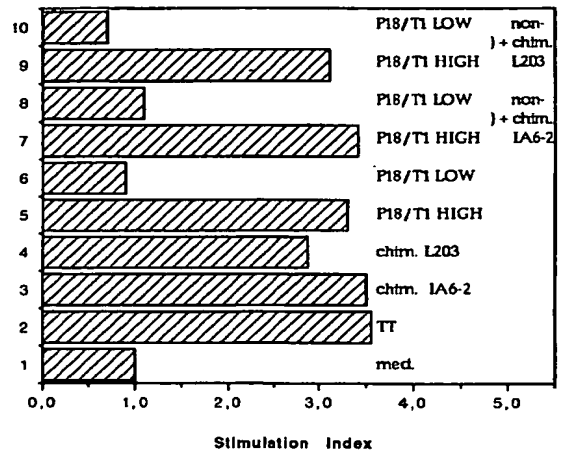

FIG. 6. Lack of polyclonal activation by immunotargeted HIV-1 peptide-expressing chimeric (chim.) Fabs. Purified PBMC (2×10^5) from HIV-1-positive donor D (see Fig. 5) were cultured for 7 days with medium (med.) alone (bar 1); a 25-μg/ml concentration of TT (bar 2), chimeric IA6-2 (bar 3), or chimeric L203 (bar 4); or a mixture of free HIV-1 peptides P18 and T1 in a large dose (each at 150 μg/ml; bars 5, 7, and 9) or a small dose (each at 1.5 μg/ml; bars 6, 8, and 10) added either alone (bars 5 and 6) or together with hybridoma-derived nonchimeric Fabs prepared by papain cleavage of the respective MAb (IA6-2 [bars 7 and 8] or L203 [bars 9 and 10]). IL-2 production was determined as described in the legend to Fig. 5. The response to TT was 6,372 cpm; that of the medium control was 1,800 cpm.

the context of the chimeric Fabs or at a high concentration (150 μg of each per ml). As seen in Fig. 6, the low free-peptide dose, when tested either alone or in combination with the native L203 or IA6-2 Fab, did not stimulate significant responses in a representative HIV-1-positive donor. The high peptide concentration stimulated a significant response which was equivalent to that induced by a 100- to 200-fold lower peptide concentration presented in the context of a chimeric immunotargeting antibody; however, this response was not augmented further by addition of the nonchimeric Fabs. These data indicate that the enhanced immunogenicity of the chimeric Fabs is not due to polyclonal APC activation and further establish the immunotargeting effect of these chimeric proteins.

As shown in Table 2, presentation of either immunotargeted chimeric Fabs or high-dose unconjugated HIV-1 peptides was significantly inhibited by an anti-CD4 MAb (OKT4) but not by an anti-CD8 MAb (OKT8). This result indicates that the peptides generated by processing of the chimeric Fab fragments are recognized in association with MHC class II molecules in a CD4-dependent fashion, similar to the corresponding synthetic peptides.

DISCUSSION

A major objective of AIDS research is development of a safe and effective vaccine capable of providing protection against a broad spectrum of HIV isolates and, possibly, also slowing down disease progression in individuals already infected (30,

TABLE 2. Effects of anti-CD4 or anti-CD8 antibodies on IL-2 production by HIV-1-positive donor-derived PBMC stimulated with chimeric Fab-immunotargeted or free HIV-1 peptides[a]

Antigen(s)	% Inhibition of IL-2 production		
	No MAb	With OKT4	With OKT8
Synthetic P18 + T1	0	78	15
Chimeric IA6-2	0	83	24
Chimeric L203	0	67	13

[a] Purified PBMC cells (2×10^5) from HIV-1-positive donor E (see Fig. 5) were cultured for 7 days with the antigens indicated (150 μg each of P18 and T1 per ml; 25 μg of chimeric Fabs IA6-2 or L203 per ml) in the absence or presence of MAb OKT4 or OKT8 (2.5 μg/ml). IL-2 production was determined as described in the legend to Fig. 5. Data are expressed as percent inhibition relative to groups with no added MAb. The responses of this donor to the corresponding antigens in the absence of an added MAb are given in Fig. 5.

39, 57). However, since HIV infection typically progresses despite the presence of a seemingly strong host immune response consisting of both neutralizing antibodies and virus-specific CTL (30), the immune response to natural infection may be relatively ineffective. The rapid (~10 days), ≤100-fold decrease in peak virus titers following primary HIV-1 infection (27) is also suggestive of a strong, but ineffective, natural immune response. On the basis of these findings, it has been argued that HIV may not follow the paradigm that underlies the successful use of vaccines against other viral infections, i.e., that the ideal vaccine should mimic as closely as possible the immune response to natural infection (26, 48). This argument has led to the suggestion that an artificial HIV subunit vaccine including only selected epitopes may be necessary (8). Many HIV-1-derived epitopes recognized by Th cells, CTL, and neutralizing antibodies have been defined. Inclusion of a Th epitope (T1; 18) and the principal neutralization domain sequence, which contains both neutralizing (37, 41) and CTL (54) epitopes, within the same synthetic peptide increased the magnitude of the HIV-specific neutralizing antibody (45) or CTL (42) response, and T1 priming generated a memory response in rhesus monkeys, evidenced by enhanced antibody production upon subsequent immunization with intact gp160 (36). The extensive strain variability among different HIV isolates presents a difficulty in the development of an effective vaccine, although many HIV isolates that exist worldwide can be grouped into five families (51) and one strain (MN) dominates among seropositive individuals tested in the United States (7). This obstacle could potentially be overcome by using a cocktail of peptides corresponding to the immunogenic but variable HIV-1 epitopes.

The underlying concept of this study is based on the notion that artificial focusing of an immunogenic peptide to APCs by an immunotargeting antibody may allow this peptide to bind at levels much higher than those expected to be achieved by physiological pathways. Thus, "forcing" the binding of a large number of homogeneous exogenous peptides to APCs by linking them to anti-MHC class II (estimated at 0.5×10^5 to 1×10^5 molecules per cell) or sIgD (2×10^5 to 5×10^5 molecules per cell; 16, 50) antibodies may increase the antigen load, thereby enhancing antigen uptake, processing, and presentation and, subsequently, T-cell activation. While the relevant studies that have documented the enhancement of immune responses by this approach (13–16, 38, 43, 47, 50) have not directly established the mechanism underlying the enhanced immune response, they have ruled out several potential mechanisms other than immunofocusing, e.g., polyclonal B-cell activation by anti-Ig antibodies or binding to Fc receptors on APCs. Although the processing pathways of these chimeric antibodies remain to be established, the fact that this experimental approach has worked successfully in a considerable number of independent studies provides a strong rationale for further exploration of its vaccine potential.

On the basis of this concept, we attempted to overcome the inherently weak immunogenicity of peptide vaccines by cloning and expressing chimeric monovalent Fab fragments of anti-human HLA-DR or sIgD MAbs that express copies of immunodominant HIV epitopes, i.e., P18 and T1. Generation of these chimeric antibodies at the gene level offers one potential advantage, i.e., the ability to attach peptide epitopes to antibody fragments under precise conditions with regard to site and copy number, thereby facilitating the preparation of multiple distinct chimeric constructs that can be compared for immunogenicity. Our results indicate that such chimeric antibodies are capable of binding to APCs and displaying the attached HIV-1 epitopes and, furthermore, that they create enhanced and specific immunogenicity by comparison with other forms of the relevant antigen (including a nonbinding chimeric Fab), as determined by their ability to stimulate IL-2 production by PBMC derived from HIV-positive donors. The immunogenicity of these synthetic HIV antigens was also demonstrated by their ability to induce Th and CTL responses in BALB/c mice upon injection into the footpads (data not shown).

Nevertheless, several potential limitations of this immunotargeting vaccine approach need to be addressed. First, bivalent immunotargeting (anti-sIg) $F(ab')_2$ fragments were found to be ~10-fold more potent than the respective monovalent Fab fragments in enhancing T-cell activation (15, 16, 47). This may reflect increased avidity of the bivalent antibody or its ability to cross-link the targeted surface molecules, i.e., MHC class II or sIg, which will, in turn, activate APCs (B cells and/or macrophages). This activation may enhance the APC antigen presentation function by upregulating adhesion molecules that facilitate interactions between T cells and APCs or by inducing secretion of immunopotentiating cytokines (44, 46). Bivalent fragments may also be favored over monovalent Fab fragments because of their longer half-life in serum (10). For these reasons, it might be advantageous to use bivalent versions of the chimeric Fab fragments to achieve improved immunotargeting in vivo. Second, the potential primate anti-mouse Ig response, which may limit the usefulness of this approach, could be addressed by creating humanized antibodies. Third, anti-MHC class II antibodies have been shown to induce immunosuppression (e.g., see reference 1). However, this effect was seen in animals treated with large quantities of antibodies over a long time period but not in successful immunotargeting studies which used single injections of the targeting anti-MHC class II antibodies (47). Thus, because of the immunopotentiating effects, a single injection (or very few injections) of immunotargeting anti-MHC class II, which is unlikely to cause immunosuppression, may be sufficient for effective vaccination.

Additional potential problems are associated with the use of anti-IgD antibodies for immunotargeting. Intravenous injection of a monovalent (but not that of a bivalent) rabbit anti-mouse IgD antibody was found to induce T-cell tolerance in mice (32, 46). Since monovalent, non-cross-linking anti-IgD antibodies failed to activate B cells, this result was interpreted as indicating that antigen presentation by resting (as opposed to activated) B cells induces T-cell anergy. On the other hand, treatment with large doses of bivalent anti-IgD can induce high levels of polyclonal IgG1 and IgE responses, as well as IL-4 production (33, 35), consistent with activation of the Th2 subset of T cells. This, in turn, would inhibit the induction of Th1 (53) cells that is considered essential for the cell-mediated immunity involved in antiviral protection (23), albeit it might potentiate the neutralizing antibody response. This mechanism was proposed to account for the suppression of cell-mediated autoimmune disease in rats treated with an anti-IgD antibody conjugated to an encephalitogenic peptide (28). However, these inhibitory effects on the induction of cell-mediated immunity are usually seen only after intravenous injection of large (100- to 1,000-μg) anti-IgD antibody doses (28, 33, 35), a procedure known to be generally effective for tolerance induction. In contrast, immunotargeting antibodies potentiated cell-mediated immunity when injected by other routes (e.g., subcutaneously) and in much smaller doses (13, 14, 38).

Concluding remarks. Chimeric anti-HLA-DR and anti-sIgD Fab fragments have been shown to act as a specific targeting device that can focus the relevant HIV-1 epitopes on the surfaces of human or primate APCs. This was demonstrated by the enhanced potency of immunotargeted antigens for presen-

tation to primed T cells over equivalent nonimmunotargeted control antigens, presumably via more efficient uptake into APCs with subsequent access to the MHC class II processing pathway, thereby leading to improved antigen presentation. Such immunotargeted chimeric constructs, which are created at the gene level and thereby allow complete control over the attachment site, copy number, and nature of the inserted HIV-1 epitopes (as opposed to the uncontrolled nature of the covalent coupling of immunogenic peptides to immunotargeting antibodies at the protein level), are potentially useful in the design of synthetic, well-characterized, and highly effective, multivalent, recombinant subunit vaccines against HIV-1. The chimeric anti-human-primate HLA-DR L203 Fab may eventually be used in nonhuman primate experiments, e.g., HIV-1 vaccine studies with *Macaca nemestrina* (2). Beyond the realm of AIDS, this approach could rapidly be translated into the generation of similar vaccine constructs targeted to other pathogens.

ACKNOWLEDGMENTS

We thank J. F. Kearney, Department of Microbiology, University of Alabama, and H. Grey for providing hybridoma IA6-2 and purified human HLA-DR, respectively, and N. Hanna (Idec, Inc., San Diego, Calif.) for providing *Cynomolgus* spleen cells. For excellent technical support, we are grateful to Leslie Giampa, Beth Herbert, and David Telford.

This work was supported in part by NIH grant AI28197 and the National Collaborative Vaccine Development Groups, NIH (U01-AI30238).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 15, Issue of April 14, pp. 8571–8577, 1995
Printed in U.S.A.
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Structure and Function of Several Anti-Dansyl Chimeric Antibodies Formed by Domain Interchanges between Human IgM and Mouse IgG2b*

(Received for publication, November 21, 1994, and in revised form, January 26, 1995)

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Two pairs of chimeric, domain-switched immunoglobulins with identical murine, anti-dansyl (5-dimethylaminonaphthalene-1-sulfonyl) variable domains have been generated, employing as parent antibodies a human IgM and a mouse IgG2b. The first pair of chimeric antibodies $\mu\mu\gamma\mu$ and $\gamma\gamma\mu\gamma$ was generated by switching the Cμ3 and Cγ2 domains between IgM and IgG2b. The second pair of chimeras $\mu\mu\gamma\gamma$ and $\gamma\gamma\mu\mu$ were formed by switching both Cμ3 and Cμ4 with Cγ2 and Cγ3. SDS-polyacrylamide gel electrophoresis and analytical ultracentrifugation showed that over half (57 and 71%) of the two chimeric antibodies possessing the Cμ4 domain and tail piece formed disulfide-linked IgM-like polymers. In contrast, the two chimeric antibodies lacking the Cμ4 domain were almost entirely monomeric. Both monomeric chimeras had reduced ability to activate complement. The chimera $\gamma\gamma\mu\gamma$ had no activity under any of the assay conditions, whereas $\mu\mu\gamma\gamma$ caused only a small amount of cell lysis but was fully active in consuming complement at 4 °C. The polymeric chimera $\gamma\gamma\mu\mu$ was much less active than IgM, bound $C\bar{1}$ weakly and caused some cell lysis but consumed little complement with soluble antigen. The polymeric chimera $\mu\mu\gamma\mu$ bound $C\bar{1}$ strongly and was the most active antibody in all assays, even more active than the parental IgG2b and IgM antibodies; it was the only antibody that exhibited antigen-independent activity. The results suggest that Cμ3 alone does not constitute the complement binding site in IgM but requires both $C\mu_{1-3}$ and $C\mu_4$ for full activity.

Complement fixation by IgG and IgM antibodies involve different mechanisms. IgG antibodies have an exposed complement binding site located in the Cγ2 domain. The participating amino acid residues, highly conserved among human, rat, mouse, guinea pig, and rabbit IgGs, include Glu^{318}, Lys^{320}, and Ly^{322} (Duncan and Winter, 1988), located on the fy3 strand of β sheet on the Fy face of the domain (Beale and Feinstein, 1976; Deisenhofer *et al.*, 1976; Edmundson *et al.*, 1975). Although C1q binds weakly to monomeric IgG (Schumaker *et al.*, 1976), strong multivalent binding to a cluster of IgG molecules attached to an antigen array is believed to be responsible for C1 activation (Hanson *et al.*, 1985; Hoekzema *et al.*, 1988; Hughes-Jones *et al.*, 1983; Metzger, 1974, 1978). This requirement for multivalent binding is referred to as the "associative" model.

IgM is a naturally occurring polymer synthesized in two forms containing either five IgM monomers and a J chain, or six IgM monomers and no J chain, arranged in a ring with the Fab's pointing outward. Each IgM monomer (μ_2L_2) is homologous in architecture to IgG, although instead of a hinge, the IgM possesses an extra domain, designated Cμ2, and is much less flexible than IgG. Although older evidence implicated the Cμ4 domain in complement fixation (Bubb and Conradie, 1978; Hurst *et al.*, 1975; Johnson and Thames, 1976), more recent studies (Arya *et al.*, 1994; Wright *et al.*, 1988) have identified residues on Cμ3 that participate in C1 binding (Arya *et al.*, 1994), and the evidence suggests that the C1 binding site is not homologous to the site on Cγ2 of IgG. Although the folding of the polypeptide backbone in Cμ3 may be modeled (Arya *et al.*, 1994), no crystallographic data are available, and the orientation of Cμ3 in the IgM molecule is unknown.

Since multiple binding sites for C1 should be present on pentameric and hexameric IgM, a single IgM attached to a red blood cell is sufficient for complement binding and activation (Borsos and Rapp, 1965a, 1965b; Ishizaka *et al.*, 1968). However, since unbound IgM binds C1q and $C\bar{1}$ weakly (Poon *et al.*, 1985; Poon and Schumaker, 1991) and does not consume complement, it is generally held that efficient interaction with C1 requires conformational changes among the IgM monomers which only occur upon interaction with antigen. A "distortional" model for complement activation in which the Fab_2 are bent toward the cell surface when IgM binds multivalently to a cluster of epitopes, converting the IgM "starfish" to the "table" or "staple" conformation with exposed C1 binding sites has been proposed (Feinstein and Munn, 1966, 1969; Feinstein *et al.*, 1983).

The technique of domain switching has been profitably employed to examine the role of the hinge and Cγ2 domain in complement fixation (Norderhaug *et al.*, 1991; Tan *et al.*, 1990; Tao *et al.*, 1991), to map the site of interaction of mouse IgE with murine FcϵRI (Weetall *et al.*, 1990), and to dissect the interaction of individual Ig domains with human Fc receptors (Shopes *et al.*, 1990). In the present study, domain exchange between a human IgM and a mouse IgG2b has been employed in a study of complement fixation and activation. A preliminary report of a similar study using mouse IgM has appeared (Chen *et al.*, 1994). The present studies indicate that the context of the C1 binding site is important in determining the efficiency of complement activation.

EXPERIMENTAL PROCEDURES

Construction of Plasmids and Production of Antibodies

The plasmid pUCγ was constructed by subcloning into pUC19 (New England BioLabs) a 3.7-kilobase pair *Xba*I-*Bgl*II fragment of genomic DNA containing Cγ1, hinge, Cγ2, and Cγ3 of mouse IgG2b. The plasmid pUCμ was generated by subcloning the 3.6-kilobase pair *Xba*I fragment of genomic DNA containing Cμ1, Cμ2, Cμ3, and Cμ4 of human IgM

* This work was supported by Research Grants GM 13914 (to V. N. S.) and CA 16858 and AI 29470 (to S. L. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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from a plasmid derived from pN-X-μTNP (Boulianne *et al.*, 1984) into pUC19. Unique *Not*I and *Mlu*I sites were generated by partial digestion of these plasmids with the appropriate restriction enzymes, isolation of singly cut plasmids by agarose gel electrophoresis, and ligation with *Not*I or *Mlu*I linkers, containing the appropriate adaptor and about 15 base pairs of the intron sequence (synthesized using the Gene Assembler, Pharmacia Biotech Inc.). A *Not*I site was introduced at the *Sac*I site in the intron 5′ to Cγ2, and an *Mlu*I was introduced at the *Ppu*MI site in the intron 3′ to Cγ2. (Fig. 1, *construct i*). *Not*I and *Mlu*I sites were introduced at the *Ppu*MI sites flanking the Cμ3 domain. (Fig. 1, *construct ii*).

The *Not*I and *Mlu*I sites were used together with the unique *Sal*I sites to generate four additional constructs, labeled *iii, iv, v,* and *vi* in Fig. 1. Each of these constructs was then joined to the mouse anti-dansyl[1] V_H from hybridoma 27–44 (Oi *et al.*, 1984) in an expression vector pSV2Hgpt-DNS-VH. Using electroporation (Shin and Morrison, 1989), the resultant vector was transfected into TSW2, an immunoglobulin nonproducing mouse myeloma cell line previously transfected with a light chain construct incorporating the κ anti-dansyl variable domain from hybridoma 27–44 joined to a human Cκ constant domain. Transfectants were selected with mycophenolic acid (Life Technologies, Inc.), and clones were screened by enzyme-linked immunosorbent assay using DNS-bovine serum albumin-coated plates and alkaline phosphatase-conjugated goat anti-human Cκ (Sigma). After subcloning, high titer antibody-producing clones were expanded in 1-liter roller bottles in Iscove's modified Dulbecco's medium containing 1% immunoglobulin-depleted calf serum (Hyclone Laboratories, Logan, UT).

Purification and Physical Analyses of Antibodies

Anti-dansyl antibodies were purified by affinity chromatography (Dangl, 1986; Schneider *et al.*, 1987) using a 1-ml column of EAH-Sepharose 4B (Pharmacia) coupled to a dansyl analog via 2-dimethylaminonaphthyl-5-sulfonyl chloride (Molecular Probes, Eugene, OR). The elution hapten, 2-dimethylaminonaphthyl-5-sulfonyl caproate, was made by overnight incubation of equal volumes of 200 mM 2-dimethylaminonaphthyl-5-sulfonyl chloride in acetone and 250 mM ε-aminocaproic acid (Sigma) in 250 mM carbonate, pH 12, and used directly at a dilution of 3 mM with phosphate-buffered saline. Eluted antibodies were collected in 1-ml fractions and the peak fractions, as judged by reducing SDS-PAGE, pooled and dialyzed in the cold with at least 24 changes of 100 × volume of TBS, pH 7.8. Antibody concentrations were calculated from A_{280}, using E (1 cm, 1 mg/ml) = 1.4 and 1.2 for mAb i and ii, respectively, and 1.3 for all chimeras.

SDS-PAGE was performed according to Laemmli (1970) using a Protean II minigel apparatus (Bio-Rad). Western blots performed as described (Burnette, 1981; Towbin *et al.*, 1979) were blotted with goat anti-human IgM (Sigma) or rabbit anti-mouse IgG2b (Miles Scientific, Naperville, IL) followed by horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG antibodies, and bands were detected using the chemiluminescence ECL reagents (Amersham Corp.).

Analytical ultracentrifugation was performed in the model E analytical ultracentrifuge (Beckman) with an UV optical system interfaced to an IBM PC computer. Sedimentation coefficients were calculated from measurements of the boundary positions at different times. The data are displayed as sedimentation distribution curves in which migrating boundaries were converted to "peaks" (Schumaker and Schachman, 1957).

Complement Assays

Direct Lysis of Dansyl-coupled SRBC—SRBC were coupled with dansylated rabbit anti-SRBC IgG(Fab′)$_2$ according to Aase and Michaelsen (1991). (Fab′)$_2$ prepared by digesting anti-SRBC antibodies (Cordis, Miami, FL) with pepsin was treated with dansyl-chloride according to Weber (1952) at an input of 23 mol/mol (Fab′)$_2$, and a dansyl$_8$-(Fab′)$_2$ was obtained based on spectral analysis (Hardy, 1986). The assay procedure was similar to that of Davis *et al.* (1988): 100 μl of SRBC, coupled with an optimal amount of the dansylated antibody fragments, at 1.5 × 10^8/ml in TBS^{++} (TBS containing 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 1 mg/ml bovine serum albumin) was incubated for 15 min at 37 °C with the indicated concentration of monoclonal antibody and then

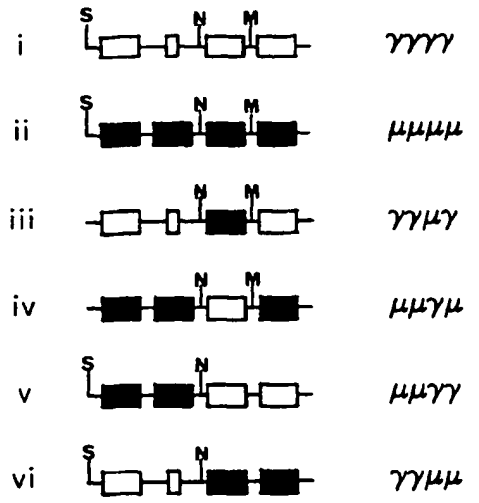

FIG. 1. **Construction of the heavy chain constant region genes for the production of chimeric antibodies.** The heavy chain exons of mouse IgG2b are represented by *empty boxes* and of human IgM by *filled boxes*. The four exons in their respective positions from *left* to *right* represent Cγ1, hinge, Cγ2, and Cγ3 of IgG2b and Cμ1, Cμ2, Cμ3, and Cμ4 of IgM. Restriction sites used for domain switching are indicated in the appropriate introns: *Not*I (*N*), *Mlu*I (*M*), and *Sal*I (*S*). To aid identification, a four Greek letter nomenclature has been used; for example, γγμγ represents the H-chain of mAb iii consisting of Cγ1-hinge-Cμ3-Cγ3.

20 μl of guinea pig serum (gpS) diluted 1/20 or normal human serum (NHS) diluted 1/10, both preadsorbed with SRBC, were added and the mixture incubated for an additional 40 min. The mixture was then diluted to 1 ml with cold TBS^{++}, centrifuged, and assayed for cell lysis by absorbance at 412 nm. Controls without mAb included tubes without serum, tubes with serum, and tubes with serum plus water as diluent.

Complement Consumption—For the soluble antigen, polylysine with an average of 560 monomer units (Sigma) was dansylated with an input of 28 mol of dansyl-chloride/mol polylysine, and approximately 17 mol of dansyl were incorporated. Consumption assays were performed according to Stollar (1978), with minor modifications. For the complement consumption step, 100 μl of TBS^{++} containing the indicated concentrations of mAb and antigen, and gpS (1/100) or NHS (1/50) was incubated either at 37 °C for 1 h or at 4 °C for 18–21 h. Then SRBC optimally sensitized with hemolysin, EA, were added, the mixture diluted to 0.7 ml (final EA concentration, 1 × 10^7 ml^{-1}), and further incubated at 37 °C for 40 min, centrifuged, and assayed for cell lysis by absorbance at 412 nm. The amounts of EA and complement had been predetermined such that, in the absence of consumption, cell lysis was 80–90% complete. Controls included tubes without mAb and tubes without antigen. Results reported for complement consumption employed a single concentration of polylysine, predetermined using fixed concentrations of mAb i and mAb ii.

RESULTS

Purification and Production of Chimeric Antibodies—Each of the gene constructs shown in Fig. 1 was used to transfect TSW2 cells, a cell line producing a chimeric light chain composed of the murine anti-dansyl variable domain and human Cκ. Antibodies were isolated from the cell culture medium by affinity chromatography, as described under "Experimental Procedures," yielding in each case 1–2 μg of purified protein/ml of culture medium. On reducing SDS-PAGE, each antibody exhibited only two bands: a 23-kDa light chain and a heavy chain whose relative size was consistent with the expected product. Western blots with anti-human-μ and anti-mouse-γ2b also indicated that domain switching had occurred in each of the chimeric antibodies (Table I).

Sedimentation coefficients and the relative amounts of monomer and polymer formed by each chimera were determined by analytical ultracentrifugation (Table I) and confirmed by non-

[1] The abbreviations used are: dansyl (or DNS), 5-dimethylaminonaphthalene-1-sulfonyl; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; SRBC, sheep red blood cells; TBS, Tris-buffered saline; gpS, guinea pig serum; gpSC, guinea pig serum complement; NHS, normal human serum; NHSC, normal human serum complement; EA, sheep red blood cells optimally sensitized with hemolysin.

TABLE I
Summary of Western blotting, ultracentrifuge, and SDS-PAGE analyses of the affinity-purified, recombinant antibodies

Treatment	Monoclonal antibody					
	γγγγ	μμμμ	γγμγ	μμγμ	μμγγ	γγμμ
a-h-μ[a]	−	+	+	+	+	+
a-m-γ2b	+	−	+	+	+	+
$s_{20,w}$	6.9		6.9	7.8 (43%)	7.7	7.2 (29%)
		18.8	10.2[c]	18.6 (57%)		17.8 (71%)
Monomer molecular mass[b]	156 kDa	190 kDa	160 kDa	184 kDa	182 kDa	163 kDa

[a] Presence (+) or absence (−) of reactivity of the corresponding immunoglobulin with either anti-human μ (a-h-μ) or antimouse γ2b (a-m-γ2b). Positive patterns were identical to the Coomassie-stained gels shown in Fig. 2*B*.
[b] Calculated as twice the molecular weights of the H-chain plus the L-chain as determined from 10% reducing SDS-PAGE.
[c] Less than 5%.

reducing SDS-PAGE (Fig. 2). The correspondence between the results obtained with the two methods indicated that the 7 S monomers and the 18 S polymers were formed by covalent inter-heavy chain disulfide bonds. In murine IgG2b, these inter-heavy chain disulfide bonds are located in the hinge, whereas in the human IgM, they were located at Cys^{337} of Cμ2, forming an intrasubunit disulfide, and at Cys^{414} of Cμ3 and Cys^{575} of the Cμ4 tail piece, forming intersubunit disulfides (Davis *et al.*, 1989b). Thus, the parental IgG2b (γγγγ) and IgM (μμμμ) were assembled as monomer and polymer, respectively, as expected. For the different chimeras, γγμγ yielded practically all monomer, although it contained Cys^{414}; μμγμ which lacked Cys^{414}, but contained Cys^{575}, yielded about 43% monomer and 57% polymer; μμγγ, possessing only the intrasubunit Cys^{337}, produced only monomer, as expected, and although γγμμ contained both Cys^{414} and Cys^{575}, it was nevertheless defective in polymer assembly, since 29% was secreted as monomer. In summary, only the mAb's containing Cμ4 with Cys^{575} present in the tail piece gave rise to appreciable amounts of polymer, with μμμμ > γγμμ > μμγμ.

Activation of Complement by Chimeric Antibodies—Since the expressed chimeric antibodies possessed identical anti-dansyl Fab, any differences in complement activation should reflect the differences in the constant regions. Differences in complement activation were studied employing both human (NHSC) and guinea pig (gpSC) complement, and complement consumption assays were performed at both 37 and 4 °C.

Direct lysis of dansyl-coupled sheep red blood cells employed a large excess of NHSC and gpSC of comparable hemolytic activity. Results shown in Fig. 3 indicate that recombinant human IgM was equally active with NHSC and gpSC, whereas recombinant mouse IgG2b was more active with gpSC. Polymeric IgM also activated complement at much lower concentrations than monomeric IgG as had been reported previously using polyclonal antibodies (Ishizaka *et al.*, 1968; van der Zee *et al.*, 1986).

For the chimeric antibodies containing Cμ3, γγμγ was inactive in the cell lysis assay, whereas γγμμ was more effective than the parental IgG2b with NHSC, but less effective than parental IgM.

For the chimeric antibodies containing Cγ2, the data show μμγμ to be more hemolytically active with both NHSC and gpSC than either parental antibody. This monoclonal antibody was 57% polymer, and presumably it was the polymer which possessed the strong lytic activity, but this was not determined. In marked contrast, the ability of the monomeric μμγγ to promote cell lysis was barely detectable.

The chimeric antibodies were also examined in an indirect assay which measured complement consumption by antibody-antigen complexes. In this assay, diluted NHSC or gpSC containing nanomolar C1 and an optimal amount (4–8 nM) of dansyl polylysine were incubated with varying concentrations (1–100 nM) of mAb. The amount of complement remaining at

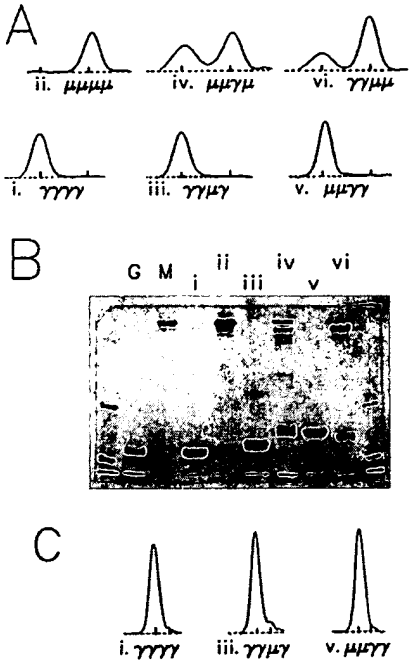

FIG. 2. **Analytical ultracentrifugation and SDS-PAGE.** *A*, sedimentation distribution patterns of affinity-purified mAb i–vi in TBS; protein concentrations ranged between 0.1 and 0.2 mg/ml. In each pattern, the *left* and *right vertical bars* denote 7 and 18 S, respectively. *B*, Coomassie Blue staining of 4% nonreducing SDS-PAGE; 15 μg of each recombinant mAb were loaded along with a nonspecific mouse IgG2b (*G*) and human IgM (*M*) and molecular mass markers of 200 and 116 kDa (flanking lanes). *C*, distribution patterns of sedimentation at high speed showing the 10.2 S component in γγμγ. In each pattern, the *left* and *right vertical bars* denote 7 and 10 S.

the end of the incubation period was then determined using a separate hemolytic assay. The results of these complement consumption assays are shown for NHSC (Fig. 4) and gpSC (Fig. 5). These data show γγμγ to be inactive in all assays, whereas γγμμ exhibited slight activity only with NHSC at 37 °C. It is noteworthy that compared with IgM, γγμμ was much less active in this consumption assay than in promoting lysis at the cell surface. μμγγ, barely active in the direct assay with both NHSC and gpSC, also was barely active in the indirect assay at 37 °C with NHSC, but showed no activity with gpSC; however, its activity was comparable with IgG2b when assayed at 4 °C. In the indirect assay, μμγμ was again the most active of all of the antibodies. Recombinant human IgM was most active with NHSC at 37 °C; in contrast, recombinant mouse IgG2b was most active with gpSC at the lower temperature. These differences were reported previously for polyclonal IgM (Cunniff and Stollar, 1968; van der Zee *et al.*, 1986) and for monoclonal IgG2b (Dangl, 1986; Gee *et al.*, 1981). Over

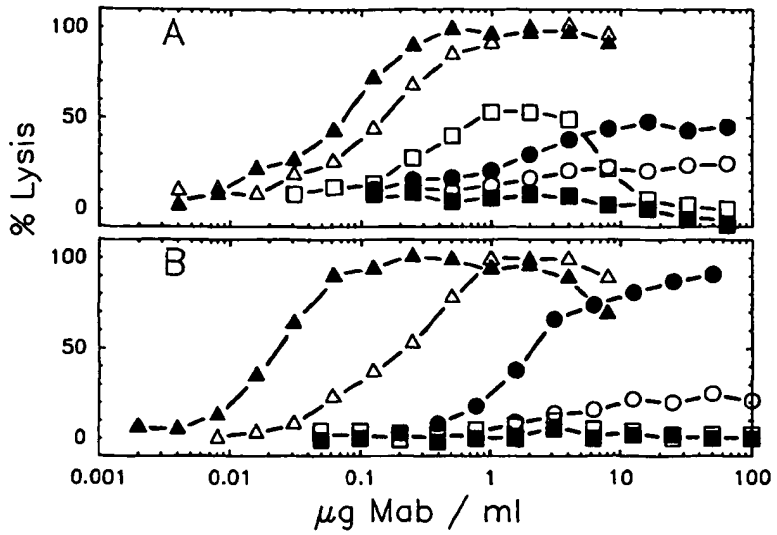

FIG. 3. **Complement activating ability of recombinant antibodies assayed by direct lysis of DNS-coupled SRBC.** *A*, using normal human serum complement; *B*, using guinea pig serum complement. 100 μl of mixtures containing 1.5 × 10^8/ml DNS-coupled SRBC and the indicated concentrations of antibodies were incubated 15 min at 37 °C, then 20 μl of diluted serum were added to a final dilution of 1/10 of NHSC or 1/20 of gpSC, and lysis was allowed to proceed for 40 min at 37 °C. The affinity-purified antibodies were mixtures of monomers and polymers, as shown in Fig. 2 and Table I: ●, IgG2b; △, IgM ; ■, γγμγ; ▲, μμγμ; ○, μμγγ; □, γγμμ.

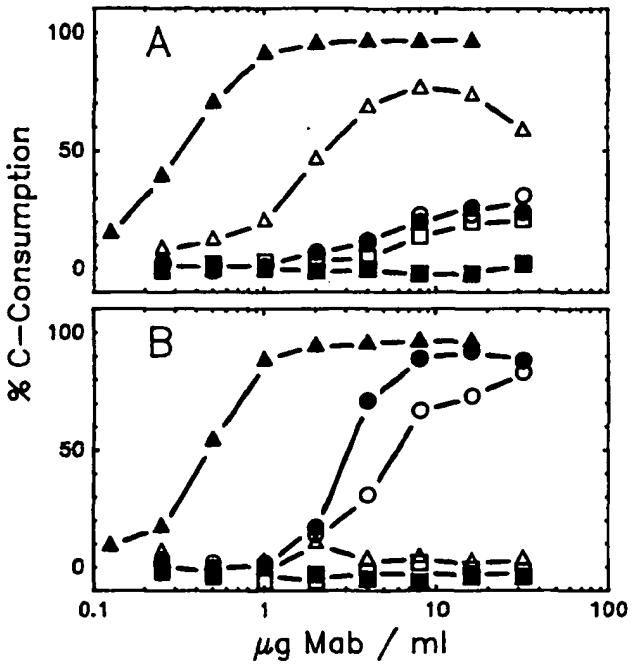

FIG. 4. **Complement consumption was assayed employing DNS_{17}-Lys_{560} as soluble antigen with normal human serum as the source of complement.** *A*, complement consumption at 37°; *B*, complement consumption at 4 °C. A 100-μl solution containing a 1/50 dilution of normal human serum together with the indicated concentration of mAb and with 8 nM DNS_{17}-Lys_{560} were incubated at 37 °C for 1 h (*A*) or with 4 nM DNS_{17}-Lys_{560} at 4 °C for 18–21 h (*B*). Then the remaining complement was assayed by addition of EA and incubation at 37 °C for 40 min. All antibodies, except μμγμ, displayed negligible activities without antigen: the results shown for μμγμ were not corrected for antigen-independent complement consumption (see Fig. 6). All antibodies were affinity-purified but represent mixtures of monomers and polymers, as shown in Fig. 2: ●, IgG2b; △, IgM; ■, γγμγ; ▲, μμγμ; ○, μμγγ; □, γγμμ.

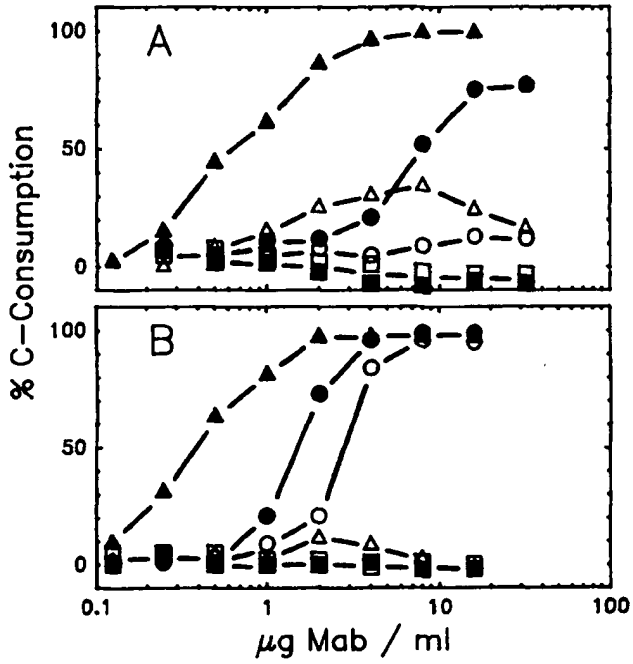

FIG. 5. **Complement consumption ability assayed employing DNS_{17}-Lys_{560} as soluble antigen with guinea pig serum as the source of complement.** *A*, complement consumption at 37°; *B*, complement consumption at 4 °C. The assay conditions were identical to those described in the legend to Fig. 4, except that a 1/100 dilution of the gpSC was used. ●, IgG2b; △, IgM; ■, γγμγ; ▲, μμγμ; ○, μμγγ; □, γγμμ.

the range of antibody concentrations employed in these assays, only μμγμ was capable of complement consumption in the absence of antigen (Fig. 6).

Ultracentrifuge Studies of the Binding of $C\bar{1}$ to the Polymeric Chimeras in the Absence of Antigen—Solutions containing human $C\bar{1}$ (0.17 mg/ml or 0.22 μM) and recombinant IgM (0.1 mg/ml or 0.11 μM polymer) or μμγμ (0.16 mg/ml or about 0.09 μM polymer) or γγμμ (0.16 mg/ml or about 0.14 μM polymer), at two ionic strengths, were studied by analytical ultracentrifugation (Fig. 7). At 0.12 M ionic strength, binding of 18.0 S $C\bar{1}$ by the 18.8 S parental IgM gave rise to a faster, broader 22 S (median) boundary, probably representing a mixture of the unassociated species reversibly equilibrating with weakly as-

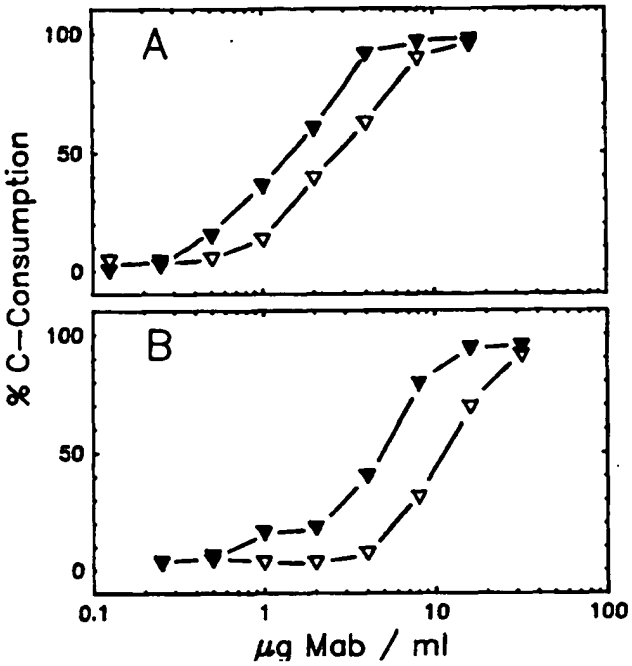

FIG. 6. **Complement consumption ability of $\mu\mu\gamma\mu$ assayed in the absence of antigens.** *A*, NHSC; *B*, gpSC. ▽, complement consumption at 37 °C; ▼, complement consumption at 4 °C. Conditions were identical to those described in the legends to Figs. 4 and 5 except that DNS_{17}-Lys_{560} was omitted.

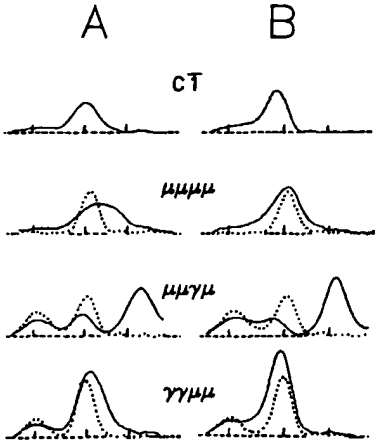

FIG. 7. **Analytical ultracentrifugation of mixtures of human $C\bar{1}$ and IgM, $\mu\mu\gamma\mu$, and $\gamma\gamma\mu\mu$ at ionic strengths of 0.120 M (*A*) and 0.150 M (*B*).** The *top panels* show $C\bar{1}$ without addition of antibody; the remaining panels represent mixtures of the indicated mAb with $C\bar{1}$ (*solid lines*) and without $C\bar{1}$ (*dotted lines*). The concentrations employed were: $C\bar{1}$, 0.17 mg/ml (0.22 μM); IgM, 0.09 mg/ml; $\mu\mu\gamma\mu$ and $\gamma\gamma\mu\mu$, 0.16 mg/ml. *Vertical bars* indicate, from *left* to *right*, 7, 18, and 27 S.

sociated 1:1 and 2:1 ($C\bar{1}$:IgM) complexes (Poon and Schumaker, 1991). When mixed with $C\bar{1}$, $\mu\mu\gamma\mu$ showed three peaks in the ultracentrifuge, probably representing the 7.8 S unassociated (trailing) monomer, the excess 18 S $C\bar{1}$, and a 30.2 S boundary probably representing a mixture of strongly associated 1:1 and 2:1 $C\bar{1}$:polymer complexes. The mixture of $\gamma\gamma\mu\mu$ and $C\bar{1}$ yielded two peaks, probably the 7.2 S unassociated monomer and a 19 S peak representing unassociated species in equilibrium with very weakly associated complexes. When the ionic strength was increased only moderately from 0.12 to 0.15 (Fig. 7*B*), the binding was practically abolished with $\gamma\gamma\mu\mu$, and much diminished with IgM, probably reflecting the 6th power dependence on ionic strength previously reported for the binding between C1q and IgM (Poon *et al.*, 1985). In addition, the decreased sedimentation coefficient for $C\bar{1}$ at the higher ionic strength (16.6 S *versus* 18.0 S) and the pronounced trailing boundary indicated that the $C\bar{1}$ complex was less stable at the higher ionic strength, probably dissociating reversibly into its 10 S C1q and 8.5 S $C1\bar{r}_2C1\bar{s}_2$ subcomponents. This enhanced dissociation of $C\bar{1}$ would have contributed to the decreased binding of $C\bar{1}$ by weakly binding IgM and $\gamma\gamma\mu\mu$. In contrast, for the mixture of $C\bar{1}$ and $\mu\mu\gamma\mu$, complex formation was almost unaffected by the increase in ionic strength, reflecting a much tighter binding between this chimera and $C\bar{1}$.

DISCUSSION

Murine IgG2b and the human IgM were employed as the parent genes for generating through exon exchange the four constructs encoding the chimeric immunoglobulins. Within experimental error, the two parental IgG2b and IgM proteins were entirely 6.9 S monomer and 18.8 S polymer, respectively, as shown by analytical ultracentrifugation and gel electrophoresis (Fig. 2). Both of these antibodies, purified by affinity chromatography, appeared uncontaminated by significant quantities of other proteins. For the parent IgM, the two intersubunit disulfides were probably formed, because mutant IgM lacking either of these have been reported partially defective in assembly (Davis *et al.*, 1989b).

The parental immunoglobulins behaved as expected in the direct lysis experiments employing sheep red blood cells coated with dansyl-coupled, anti-sheep $(Fab')_2$ and guinea pig complement. The IgM parent achieved 50% cell lysis at 0.2 μg/ml IgM, whereas the IgG2b parent reached 50% at about 3 μg/ml of protein. The greater efficiency of IgM compared with IgG on a weight basis in cell lysis probably reflects the requirement for only a single IgM to establish a complement activation site, whereas at least two IgG must be bound in close proximity (Borsos and Rapp, 1965a, 1965b; Ishizaka *et al.*, 1968). In cell lysis experiments, not only must complement be bound and activated, but additional interactions between antibody and C4b appear to be critical as well (Ziccardi, 1986); clearly both parental antibodies were capable of supporting all the required reactions.

The parental immunoglobulins also behaved as expected in the consumption assays employing soluble dansylated polylysine as antigen. The mechanisms involved in consumption have not been fully clarified; however, in contrast to the direct lysis experiments where only a small fraction of the added complement need be activated to cause cell lysis, the complement consumption experiments required that a significant fraction of the added complement be consumed for the difference to be measured. Roughly equivalent amounts of IgM antibody and complement C1 were added in these experiments, that is, 100 μl of a 1/100 dilution of serum contained about 2 pmol of C1, and 100 μl of 1 μg/ml IgM contained about 1.1 pmol. Thus, if each IgM tightly bound a single molecule of C1 rendering it unavailable for the subsequent lysis experiments, about 50% of the complement would have been "fixed" or consumed; C1 binding may have been the principle mechanism of complement fixation at 4 °C where activation of C1 is expected to be minimal. Alternatively, and especially at 37 °C, the C1 could have been activated, leading to activation and depletion of essential components of the classical pathway subsequently employed in the lytic step of the assay. It has been observed previously that 7 S antibodies react much more effectively at 4 °C than at 37 °C, whereas 19 S antibodies are relatively more reactive at 37 °C than at 4 °C (Cunniff and Stollar, 1968). Our results for the parental immunoglobulins are in complete agreement with these earlier studies, for it was found that the IgM parent

consumed complement much more effectively at 37 °C than at 4 °C, whereas for the IgG2b parent, the converse was true, (Figs. 4 and 5).

Monoclonal antibody $\gamma\gamma\mu\gamma$ was approximately 95% monomer and only 5% dimer (Table I), even though the Cμ3 domain contained Cys^{414} used for one of the two intersubunit disulfide bridges found in wild-type IgM polymer. In the mutant IgM-Ser^{575}, Cys^{414} was used to produce the 20–40% pentamer/hexamer observed (Davis *et al.*, 1989a). Absence of polymer formation with $\gamma\gamma\mu\gamma$ may reflect the lack of the tail piece, which appears to play an essential role in polymer formation (Baker *et al.*, 1986; Davis *et al.*, 1988, 1989a). However, IgG3 lacking a tail piece but mutated to contain Cys^{309} (corresponding by sequence homology to Cys^{414} in Cμ3), although secreted predominately as monomer, formed a mixed population of oligomeric antibodies ranging in size from monomer to hexamer (Smith and Morrison, 1994).

Mutant $\gamma\gamma\mu\gamma$ was the only one of the six antibodies studied that was completely unable to activate or consume complement in either the direct or indirect assays (Figs. 3–5) as had previously been observed with a similar $\gamma\gamma\mu\gamma$ chimera (Chen *et al.*, 1994). Upon aggregation by antigen, this monomeric antibody would be expected to bind and activate complement if a complement binding site were exposed on the Fc. It would appear that the failure to activate complement reflected the lack of a functional complement binding site on the Cμ3 domain.

Chimera $\mu\mu\gamma\mu$ which lacked Cys^{414} of IgM which participates in the intersubunit bonds was secreted as 57% polymer and 43% monomer. The amount of polymer was somewhat higher than might have been expected given that the mutant IgM-Ser^{414} secreted only 20–40% polymer (Davis *et al.*, 1989b). Mutant $\mu\mu\gamma\mu$ was more active than either parental antibody in both the direct and indirect complement assays and was the only monoclonal that was active in the absence of antigen (Fig. 6). This last observation implies that the complement binding site on Cγ2 must have been exposed in the absence of antigen on the IgM-like polymer. Moreover, comparison of Figs. 4 and 5 with Fig. 6 shows that the activity was increased 4–20-fold upon addition of dansylated polylysine. It seems possible that the IgM architecture, especially Cμ2 and Cμ4, partially concealed the complement binding site, and that full exposure was restored when $\mu\mu\gamma\mu$ was distorted upon multivalent interaction with the dansylated polylysine.

In contrast to murine IgM, which requires the intersubunit disulfide bonds linking Cys^{414} residues for significant complement activity (Davis *et al.*, 1989b), the presence of these disulfides clearly was not essential for the strong complement activity displayed by chimera $\mu\mu\gamma\mu$. This result is similar to what is observed for polymeric IgG3 containing the IgM tail piece (Smith and Morrison, 1994), in which introduction of the analogous disulfides neither enhanced nor inhibited activity.

Chimera $\mu\mu\gamma\gamma$ which lacked the tail piece and both Cys^{414} and Cys^{575}, and was secreted only as a 7.2 S monomer, did not cause significant cell lysis unless high concentrations were employed (Fig. 3). However at 4 °C, this antibody consumed complement almost as well as IgG2b. These results were surprising, since $\mu\mu\gamma\gamma$ contained the same preformed complement binding site on Cγ2 as the monomeric IgG2b and presumably the polymeric form of $\mu\mu\gamma\mu$, both of which effectively lysed red blood cells. Perhaps monomeric chimera $\mu\mu\gamma\gamma$ bound C1 but failed to promote other steps essential for the lytic chain.

The high polymer content (71%) of $\gamma\gamma\mu\mu$ probably reflected the availability of both Cys^{414} and Cys^{575} for intersubunit disulfide bridges. However, since the remaining 29% of this protein was secreted as monomer, it may be concluded that domain interactions involving Cμ1 and Cμ2 in addition to those involving Cμ3 and Cμ4 were involved in the complete assembly of the IgM polymer. Chimera $\gamma\gamma\mu\mu$ promoted cell lysis (Fig. 3) in the presence of normal human serum, but the curve reached a maximum at 50% lysis, then leveled off and finally dropped toward zero, probably reflecting the transition between polyvalent and monovalent binding expected to occur at high antibody concentrations (Pruul and Leon, 1978). This phenomenon was also observed for complement consumption by the parental IgM (Figs. 4*A* and 5*A*). Chimera $\gamma\gamma\mu\mu$ was about an order of magnitude less effective than the parental IgM in promoting cell lysis with human serum, and much less complement consumption was found for this chimera with the soluble dansylated polylysine antigen, possibly reflecting a defect in the stabilization of an activating conformation because of the substitution of an intact Cμ2 domain by an IgG hinge. A similar conclusion was reached in a study of the effects of limited denaturation of Cμ2 in equine IgM by heat (Siegel and Cathou, 1981).

Binding affinities between the polymeric chimeras and $C\bar{1}$ were reflected by the enhanced sedimentation coefficients of the leading peaks observed during the ultracentrifuge analyses (Fig. 7), the affinity for $C\bar{1}$ being greatest for $\mu\mu\gamma\mu$, followed by IgM and weakest for $\gamma\gamma\mu\mu$. It is interesting that this ranking also corresponds to their abilities to activate complement (Figs. 3 and 4). The sedimentation data also give information about the size of the complexes. In a previous report (Poon and Schumaker, 1991) the 1:1 $C\bar{1}$:IgM complex had a measured sedimentation coefficient of 27 S. Therefore, the 22 and 19 S peaks observed when $C\bar{1}$ was added to IgM and $\gamma\gamma\mu\mu$, respectively, probably represented a dynamic equilibrium between the unassociated species and the predominantly 1:1 complexes. A 2:1 complex of $C\bar{1}$:IgM should have a sedimentation rate of 35–36 S. Therefore, the 30 S complex shown in Fig. 7*A* for the leading peak in mixtures of chimera $\mu\mu\gamma\mu$ and $C\bar{1}$ probably represents a dynamic equilibrium between the unassociated species with predominately 1:1 and 2:1 complexes of $C\bar{1}$:$\mu\mu\gamma\mu$. Previously it was shown that 2:1 complexes were formed when high concentrations of C1q were added to IgM, and when these were cross-linked and visualized with the electron microscope, C1q was seen to be bound to opposite sides of the IgM disk (Poon *et al.*, 1985).

What conclusions may be drawn from these studies concerning the mechanism of complement binding and activation and the functions of the various domains? First, the data support an associative model for complement activation for IgG, for the cluster of Cγ2 presented by chimera $\mu\mu\gamma\mu$ was sufficient to bind C1 strongly and activate complement even in the absence of antigen. As a corollary, it follows that the complement binding site must be at least partially exposed when Cγ2 is introduced into the IgM architecture. Moreover, the data also show that full exposure or proper orientation of the C1 binding sites was induced by multivalent binding of chimera $\mu\mu\gamma\mu$ to dansylpolylysine, which can support the distortive model for complement activation by IgM, although additional aggregation may contribute to the enhanced activation. Cμ1 and Cμ2 probably transmitted this distortion in wild-type IgM; however, when they were replaced by Cγ1 and the hinge in $\gamma\gamma\mu\mu$, activity was greatly diminished. Although assembly of IgM monomer to form polymer required the tail piece and at least one of the two intersubunit disulfides, Cμ1 and Cμ2 must also play some role in assembly, because 29% of the protein was secreted as monomer in their absence. In addition, Cμ3 did not appear to contain a complete complement binding site, for chimera $\gamma\gamma\mu\gamma$ was inactive in all of the assays. Probably Cμ4 must act together with Cμ3 to form the binding site; alternatively, it is possible that adjacent Cμ3 domains joined by the Cys^{414} disul-

fide form the site, or the γ domains render a site on Cμ3 inaccessible. Finally, we should point out that these antibodies were chimeras not only of Cμ and Cγ domains, but also of human and mouse proteins. Interpretation of these data are probably not seriously limited by the cross-species composition, for the divergence in primary sequence between IgM and IgG class antibodies is greater than the cross-species divergence between immunoglobulins of the same class.

The results presented here coupled with previous studies allow us to formulate a model for complement binding by IgM. In the absence of antigen, IgM has been shown to bind two molecules of C1q, one on each side of the symmetrical IgM disk, in a noncooperative manner (Poon *et al.*, 1985). That the binding was noncooperative would seem to rule out a two-state model in which the IgM flickers between a binding and a nonbinding conformation, since in that case, the first bound C1q would assist in maintaining the binding conformation for the second C1q, yielding a cooperative interaction. Noncooperative binding implies an alternative model in which weak, independent binding sites for C1q were always exposed on both sides of the IgM disk.

If weak binding sites were always present, then the required conformational change probably aligned additional residues to form a tight binding site. Residues on both Cμ3 and Cμ4 may be required to form a binding site, since chimera γγμμ was capable of erythrocyte lysis, whereas chimera γγμγ was totally inactive. Cμ2 probably transmits the distortion in IgM occasioned by multivalent binding, possibly rotating Cμ3 with respect to Cμ4 to convert a weak to a strong complement binding site.

Although this low resolution model contains speculative elements, it may prove useful in the design of further experiments to locate the complement binding site and elucidate the conformational change required for the activation of complement by IgM.

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Phase I Clinical Trial Using Escalating Single-Dose Infusion of Chimeric Anti-CD20 Monoclonal Antibody (IDEC-C2B8) in Patients With Recurrent B-Cell Lymphoma

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The B-cell antigen CD20 is expressed on normal B cells and by nearly all B-cell lymphomas. This nonmodulating antigen provides an excellent target for antibody-directed therapies. A chimeric anti-CD20 antibody (IDEC-C2B8), consisting of human IgG1-κ constant regions and variable regions from the murine monoclonal anti-CD20 antibody IDEC-2B8, has been produced for clinical trials. It lyses CD20$^+$ cells in vitro via complement and antibody-dependent cell-mediated lysis. Preclinical studies have shown that the chimeric antibody selectively depletes B cells in blood and lymph nodes in macaque monkeys. In this phase I clinical trial, 15 patients (3 per dose level) with relapsed low-grade B-cell lymphoma were treated with a single dose (10, 50, 100, 250, or 500 mg/m^2) of antibody administered intravenously. Treatment-related symptoms correlated with the number of circulating CD20 cells and grade II events consisted of fever (5 patients), nausea (2), rigor (2), orthostatic hypotension (2), bronchospasm (1), and thrombocytopenia (1). No significant toxicities were observed during the 3 months of follow-up. Serum C3, IgG, and IgM levels, neutrophils, and T cells were largely unchanged. At the three higher dose levels, pharmacokinetics of the free antibody showed a serum half-life of 4.4 days (range, 1.6 to 10.5). Levels greater than 10 μg/mL persisted in 6 of 9 patients for more than 14 days. No quantifiable immune responses to the infused antibody have been detected. CD20$^+$ B cells were rapidly and specifically depleted in the peripheral blood at 24 to 72 hours and remained depleted for at least 2 to 3 months in most patients. Two-week postinfusion tumor biopsies showed the chimeric antibody bound to tumor cells and a decrease in the percentage of B cells. Tumor regressions occurred in 6 of 15 patients (2 partial and 4 minor responses). The results of this single-dose trial have been used to design a multiple-dose phase I/II study.

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THE TREATMENT OF patients with relapsed non-Hodgkin's lymphoma (NHL) remains a frustrating problem. More than 50% of patients with aggressive lymphomas and the majority of patients with low-grade lymphomas are not cured by current therapies. New treatments with different mechanisms of action and toxicity profiles are needed. Previous work using patient-specific anti-idiotype monoclonal antibodies (MoAbs) has shown that NHLs are accessible to intravenously infused antibody, and that tumor regressions, including durable complete remissions, can be induced in some patients.[1-3] With current technology, because of the difficulty and time required to produce patient-specific antibodies, this approach is not feasible for general application. In contrast, the antigen CD20, a 32-kD nonglycosylated phosphoprotein present on the surface of nearly all B cells provides a more universal target for immunotherapy.[4] CD20 is expressed on the surface of normal B cells from the time of cytoplasmic μH chain expression throughout differentiation until the antibody-secreting plasma cell stage. Importantly, it is not expressed on early pre-B cells, stem cells, or antigen-presenting dendritic reticulum cells.[5] More than 90% of B-cell NHLs express this surface protein.[6,7] It is also expressed at a lower density on B-cell chronic lymphocytic leukemia.[8] Although the function of this molecule is not completely defined, it may aggregate and function as a calcium channel.[9] Antibodies binding to surface CD20 can induce a transmembrane signal[10] that can cause a variety of effects from cell activation to blocking cell cycle progression and differentiation.[11,12]

The CD20 protein has multiple *trans*-membrane domains and does not modulate from the cell surface in response to antibody binding and thus provides an ideal target for immunotherapeutic strategies not depending on internalization for their antitumor effect. Unconjugated murine MoAbs to CD20 have been used for immunotherapy. A trial of the 1F5 murine IgG2a anti-CD20 MoAb in four patients showed antitumor activity with minimal toxicity at the highest dose level.[13] Two trials have been reported using anti-CD20 radioimmunoconjugates. Bone marrow ablative doses of ^{131}I-conjugated B1 (IgG2a murine MoAb anti-CD20) resulted in complete remissions in 84% of patients.[14] The use of non–marrow-ablative doses also resulted in partial and complete remissions in the majority of patients.[15] In both of these trials, antitumor effects were observed during the imaging portion of the trials when trace doses of radiolabeled MoAbs were infused with large amounts of unlabeled antibody, suggesting that the murine anti-CD20 MoAb itself may be contributing to the antitumor effect. Indeed, the relative contributions to the antitumor effect of the targeted radiotherapy, of the cold antibody, and of the nonspecific whole body radiation delivered by the radioimmunoconjugates is difficult to determine.[16,17]

Murine MoAbs have several potential limitations when used in clinical trials. Genetic manipulation has made it possible to engineer chimeric antibodies with murine binding

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Submitted February 9, 1994; accepted June 10, 1994.

Supported in part by Grant No. CA34233 from the US Public Health Services, National Institutes of Health. D.G.M. is supported by a Clinical Associate Physician Award from the General Clinical Research Center. R.L. is an American Cancer Society Clinical Research Professor.

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0006-4971/94/8408-0013$3.00/0

sites and human constant regions that have lower immunogenicity, longer half-life, and are able to lyse tumor cells using human complement or antibody-dependent cell-mediated cytotoxicity (ADCC) effector cells more effectively than the murine MoAb.[18-20] A chimeric anti-CD20 antibody has been produced that contains the heavy and light chain variable regions from a murine IgG1 monoclonal anti-CD20 antibody (IDEC-2B8) and human IgG1 κ constant regions.[21] Stable, high-level expression was obtained by transfection of the relevant gene constructs into Chinese hamster ovary (CHO) cells. In vitro studies showed similar binding characteristics between the chimeric and murine anti-CD20 antibodies; however, the chimeric antibody was able to lyse $CD20^+$ B cells using human complement or human effector cells (ADCC) 1,000-fold more effectively than the murine antibody. Preclinical studies in macaque cynomolgus monkeys have shown that repeated doses of the chimeric antibody depleted around 80% of $CD20^+$ B cells in the peripheral blood, lymph nodes, spleen, and bone marrow, with gradual recovery over a period of several months.[21] No toxicity was observed in these studies. We describe here the first phase I clinical trial of single-dose infusion with the chimeric anti-CD20 antibody (IDEC-C2B8) in patients with relapsed B-cell NHL.

MATERIALS AND METHODS

Chimeric monoclonal anti-CD20 antibody. The chimeric monoclonal anti-CD20 antibody (IDEC-C2B8) has been produced and provided for clinical trials by IDEC Pharmaceuticals Co (San Diego, CA) and supplied under an Investigational New Drug Application.

Protocol design. This was a phase I clinical trial of single-dose IDEC-C2B8 chimeric anti-CD20 MoAb administered to patients with relapsed B-cell NHL. Detailed informed consent was obtained from all patients in accordance with the human subjects institutional review board of Stanford University Medical Center. Three patients were treated at each dose level with a single intravenous infusion of 10, 50, 100, 250, or 500 mg/m^2 of MoAb. Patients were evaluated for infusional related toxicity and effect on peripheral blood B cells, T cells, neutrophils and platelets, serum chemistries, Ig, and complement levels. In patients treated at the upper three doses, tumor biopsies were obtained 2 weeks after treatment and examined for evidence of antibody binding and B- and T-cell content. All patients were evaluated for antitumor activity.

Patient selection. On entry to the study, patients were required to have relapsed NHL with measurable disease after at least one prior course of standard therapy. A tumor biopsy was performed to document tumor cell expression of the CD20 antigen and reactivity with IDEC-2B8 or IDEC-C2B8 antibodies using flow cytometry. In addition, baseline hematologic function (1,500 granulocytes and 50,000 platelets/μL), renal function (serum creatinine of <2.5 mg/dL), quantitative serum IgG of greater than 600 mg/dL, a negative serology to human immunodeficiency virus, a negative hepatitis B surface antigen, and a life expectancy of at least 3 months without other serious illness was required. Patients previously exposed to murine antibodies were required to have no evidence of a pretreatment human antimurine antibody immune response (HAMA).

Flow cytometry. CD20 antigen expression was determined on all cases before antibody treatment by flow cytometry of fresh or cryopreserved tumor cell suspensions. Tumor cells were obtained from excisional biopsies or from fine needle tumor aspirations and stained for CD20 expression with fluorescein isothiocyanate (FITC)-conjugated IDEC-2B8 or IDEC-C2B8 (IDEC Pharmaceuticals) and Leu-16, an independent anti-CD20 antibody (Becton Dickinson, San Jose, CA). Tumor cells were also analyzed for expression of surface Ig light chains [FITC-goat F(ab)$_2$-antihuman κ or λ; Tago, Burlingame, CA), CD19, CD4, CD3, CD8 (FITC- or phycoerythrin [PE]-conjugated Leu12, Leu3, Leu4, and Leu2; Becton Dickinson), and CD37 (MB1 clone 6A4). Peripheral blood samples were analyzed for the number of cells expressing the CD20 antigen using two-color flow cytometry using PE or FITC conjugates of the above reagents.

Two-week posttreatment tumor biopsies were also evaluated for B- and T-cell content using the same reagents described above. Antibody bound to tumor cells from in vivo administration was detected by a combination of two different methods. In the first method, cells were stained using FITC-labeled anti-CD20 antibodies. The presence of the unlabeled antibody blocked the binding of the labeled antibody, resulting in decreased immunostaining of the B-cell tumor population (as identified using antibodies to additional B-cell antigens CD19, CD37, IgM, IgG, κ or λ). Second, the bound chimeric antibody was detected directly by looking for IgM κ- or λ-positive tumor cells now bearing the human IgG (κ) constant regions of the chimeric antibody (IDEC-C2B8) using an FITC-labeled goat F(ab)$_2$ antihuman IgG γ-chain–specific reagent (Tago). An estimate of the percentage of tumor cells with the chimeric antibody attached was obtained by comparing the staining of the pretreatment and the posttreatment biopsies for human IgG constant regions.

IDEC-C2B8 pharmacokinetics. Serum levels of the chimeric antibody were determined using an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with a purified polyclonal goat anti-IDEC C2B8 idiotype antiserum. After washing and blocking, posttreatment sera were serially diluted. Bound human IgG was then detected using a horseradish peroxidase (HRP)-conjugated polyclonal antihuman IgG reagent, and the plates developed with the substrate 2,2-azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS). Antibody concentration was determined by comparison of the signal from the patients sera with that obtained from known concentrations of purified chimeric antibody diluted into normal human serum.

Measurement of host anti-IDEC C2B8 antibody response. Posttreatment sera from evaluations at 1, 2, and 3 months were analyzed for evidence of a host antichimeric antibody immune response using a sandwich ELISA with microtiter plates coated with IDEC C2B8, the murine antibody 2B8, or normal murine IgG. Dilution's of the patients sera were added and, after washing, detected with biotin-labeled IDEC C2B8 followed by Avidin-HRP and the substrate ABTS. This assay has a level of quantification of 5 μg/mL.

Study measurements. Patients were evaluated for infusional related toxicity using the National Cancer Institute's Common Toxicity Criteria. Hematologic, renal, and hepatic function was monitored before and after infusion and during monthly intervals after therapy. Sera for evaluation of antibody levels and pharmacokinetics, serum IgG and IgM levels, and CD20 expression on peripheral blood B cells was obtained at each follow-up visit. Tumor response was assessed by evaluation of tumor measurements from physical examination and from radiologic imaging studies. For 3 months after therapy, patients were evaluated at monthly intervals and then followed at 1- to 3-month intervals until disease progression was observed. A complete remission (CR) required complete resolution of all detectable disease. A partial remission (PR) required a greater than 50% reduction in measurable disease persisting more than 30 days. A minor response (MR) was defined as a 25% to 50% reduction in disease. Stable disease (SD) was defined as no significant change in tumor measurements without progression over the period of observa-

Table 1. Patient Characteristics

Patient No./Sex/Age	Dose (mg/m^2)	Tumor Histology	Stage*	Prior Therapy	Disease Bulk†	Maximal Response
001/M/53	10	FM	III	Splenectomy C-MOPP m-BACOD CHOP	+++ Leukemia	
002/M/55	10	FSC	III	XRT (total nodal) CVP Chl	+ Leukemia Splenomegaly	Delayed PR?
003/M/49	10	DILD (mantle zone)	IV	MACOP-B Chl Anti-Id (MoAb) CVP	++	
004/M/63	50	FSC DLC FM	IV	CVP Id-Vac ProMACE/CytaBOM	+	Mixed
005/M/61	50	FSC	III	XRT anti-Lym-1 (MoAb) Chl/P CVP	++	
006/F/59	50	SL	IV	CVP CVP CVP/BVP	+++	
007/F/58	100	DLC, monocytoid B cell	IV	MACOP-B XRT	+ Splenomegaly	PR
008/M/73	100	FM	IV	XRT Chl anti-CD4 (MoAb)	++	MR
009/M/65	100	FM	IV	Chl/P Splenectomy	++	Mixed
010/M/38	250	FSC (diffuse areas)	IV	Chl Velban CVP Anti-Id (IFN) Anti-Id (Chl) MINE/ESHAP ^{90}Y-B1	++ Splenomegaly	
011/M/46	250	DLID (mantle zone)	IV	CVP	+++ Splenomegaly	MR
012/F/52	250	FSC	IV	CHOP/XRT Chl/P Chl/P VACOP-B Fludarabine IFN	+++	
013/M/48	500	FM	IVb	CHOP DHAP CVP ^{90}Y-B1	+	MR
014/F/65	500	FM	IV	CHOP Ctx	+	MR
015/F/38	500	FSC	IV	ProMACE/MOPP XRT IFN	+	PR

Abbreviations: FSC, follicular small cleaved cell; FM, follicular mixed small and large cell; DLID, diffuse lymphoma intermediate differentiation; DLC, diffuse large cell; SL, small lymphocytic; XRT, radiation therapy; IFN, interferon α; ProMACE/MOPP, etoposide, cyclophosphamide, adriamycin, methotrexate, prednisone, nitrogen mustard, vincristine, and procarbazine; ProMACE/CytaBOM, cyclophosphamide, adriamycin, etoposide, prednisone, cytarabine, bleomycin, vincristine, and methotrexate; Ctx, cyclophosphamide; CHOP, cyclophosphamide, adriamycin, vincristine, and prednisone; CVP, cyclophosphamide, vincristine, and prednisone; ^{90}Y-B1, ytrium-labeled B1 (anti-CD20 MoAb); DHAP, cisplatin, cytarabine, and decadron; VACOP-B, VP-16, adriamycin, cyclophosphamide, vincristine, prednisone, and bleomycin; Chl/P, chlorambucil and prednisone; MINE-ESHAP, ifosphamide, novantrone, etoposide, platinum, and cytarabine; Anti-Id, anti-idiotype MoAb; Id-Vac, Idiotype vaccination; BVP, bleomycin, vincristine, and prednisone; Mixed, regression noted in some but not all areas.

* Clinical stage at disease diagnosis.

† Tumor bulk estimated from physical exam and CT scans of the chest, abdomen, and pelvis and graded as follows: multiple areas of adenopathy with largest mass <5 cm (+), nodal mass >5 cm (++), extensive disease with multiple areas >5 cm (+++).

tion. Progressive disease (PD) was noted when there was a 25% increase in measurable disease or the appearance of any new lesion.

RESULTS

Patient characteristics. Fifteen patients were entered into the study. The characteristics of these patients are shown in Table 1. Patients ranged in age from 38 to 73 years, with a mean age of 55 years. Fourteen of the patients had an initial tumor diagnosis of a low-grade histology, with 6 being predominately follicular small cleaved cell, 5 being follicular mixed small cleaved and large cell, 2 being diffuse intermediate cell (mantle zone), and 1 being small lymphocytic

Table 2. Infusional-Related Symptoms During Chimeric Anti-CD20 Antibody Therapy

Patient No.	Dose (mg/m²)	Dose given (mg)	Pre Rx CD20 (cells/μL)	Toxicity
001	10	16	1,630 (tumor)	Fever, rigor, bronchospasm
002	10	22	960 (tumor)	Fever, rigor, nausea
003	10	21	50	None
004	50	100	60	Fever
005	50	100	40	Fever, chill
006	50	90	140	Myalgia
007	100	180	130	Fever
008	100	188	50	Fever
009	100	200	580 (normal B)	Fever, rigor
010	250	520	90	Chill
011	250	575	130	Fever, headache
012	250	400	20	Fever
013	500	1,200	60	Fever orthostatic hypot. nausea
014	500	965	130	Fever, nausea
015	500	760	160	Fever, nausea, headache

lymphoma. One patient had an initial diagnosis of a diffuse large-cell lymphoma; however, a relapsed node biopsy showed a monocytoid B-cell histology. Two patients with an initial diagnosis of a low-grade lymphoma had histologic progression on tumor biopsies performed before antibody therapy (1 follicular large cell and 1 diffuse large cell). The majority of patients (12/15) presented with stage IV disease. Patients had lived with their disease a mean of 5 years (range, 1.7 to 9.5) at the time of antibody therapy. All patients had measurable progressive disease and had received a median of two prior regimens (range, 1 to 5) of conventional therapy within the past 1.5 years. Five patients had previously been treated with murine MoAbs, including 2 patients who had received radiolabeled anti-CD20 antibody therapy (1 with a short PR and 1 with no response). All patients previously exposed to murine antibodies were HAMA-negative at the time of treatment.

Infusional-related toxicity. All patients completed the planned antibody infusion. Infusional-related symptoms are detailed in Table 2. The most frequent side effect was low-grade fever, which was seen in 13 of 15 patients (grade II in 5/15). Three patients additionally developed rigors and 1 developed bronchospasm requiring transient administration of supplemental oxygen. One patient on multiple medications for high blood pressure developed orthostatic hypotension during the antibody administration. In all patients, the antibody infusion was temporarily discontinued when significant side effects were observed. If necessary, the patients were treated as indicated with diphenhydramine and acetaminophen. Antibody infusions were usually restarted within 30 to 45 minutes at 50 to 100 mg/h and then escalated as tolerated to 200 mg/h. No significant further toxicity except fever was observed in any patients during the remainder of the antibody infusion. Three of the 4 patients having more significant reactions were also noted to have the highest levels of pretreatment $CD20^+$ cells in the peripheral blood (Table 2). Interestingly, these cells could be malignant (patients no. 1 and 2) or normal (mixed κ or λ phenotype, as observed in patient no. 9).

Effect on circulating B cells. In all patients, the numbers of B cells present in the peripheral blood before and after anti-CD20 therapy were analyzed using two-color flow cytometry. B cells were identified using the B-cell antigens CD19 and surface Ig, neither of which is blocked by the binding of the chimeric antibody to CD20. Bound antibody from in vivo administration was identified on the B cells by finding human IgG or κ bound to B cells coexpressing IgM and λ, or by the blocking of binding of a directly labeled anti-CD20 antibody. There was a dose-dependent, rapid, and specific depletion of the B cells in all patients, especially those receiving doses of more than 100 mg. In all but 1 patient receiving the higher doses (>50 mg/m²), these depletions persisted for 1 to greater than 3 months. The specificity of the B-cell depletion in a patient receiving 100 mg/m² is shown in Fig 1. After intravenous administration of 180 mg of antibody, there was a rapid and complete disappearance of B cells expressing CD20, CD19, κ, or λ surface antigens. There was no effect on the number of T cells (CD3), and the decrease in the numbers of lymphocytes is accounted for entirely by the decrease in the numbers of B cells. Peripheral blood B-cell levels for the duration of the 3-month study are shown on all 15 patients in Table 3. Three patients had circulating tumor cells, as shown by a clonal expansion of cells containing the same heavy and light chain isotype as identified on cells of their lymphomas obtained from tumor biopsies. Three patients had no detectable peripheral blood B cells at the 3-month evaluation. Follow-up is available

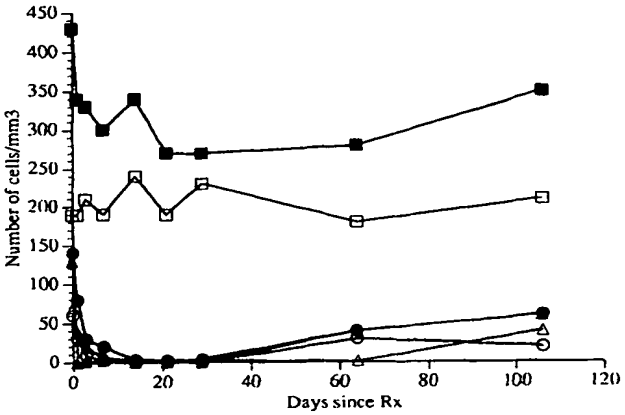

Fig 1. Depletion of peripheral blood B cells after 100 mg/m² chimeric anti-CD20 antibody. Patient no. 007 was treated with 100 mg/m² of chimeric anti-CD20 administered intravenously on day 1. Peripheral blood mononuclear cells were analyzed by flow cytometry for the expression of B- and T-cell antigens. There was a rapid and specific depletion of B cells, as indicated by the loss of cells expressing κ or λ light chains as well as the B-cell antigens CD19 and CD20. (■) Lym; (●) CD19; (▲) CD20; (□) CD3; (△) K; (○) L.

Table 3. Depletion of B Cells From the Blood After Infusion of Chimeric Anti-CD20 Antibody

Patient No.	Dose (mg/m²)	Pre Rx CD19 (cells/μL)	B-Cell Depletion Post-Rx CD19				
			d 3	1 wk	1 mo	2 mo	3 mo
001	10	1,940	995	1,260	ND	ND	ND
002	10	2,240	1,250	1,870	2,150	1,370	1,270
003	10	60	10	10	75	180	290
004	50	60	6	5	5	ND	ND
005	50	50	3	1	6	ND	ND
006	50	80	130	80	50	90	110
007	100	140	30	20	4	40	60
008	100	40	10	1	8	8	10
009	100	600	5	9	10	6	130
010	250	90	1	0	10	0	0
011	250	140	180	165	120	120	ND
012	250	20	5	0	3	0	0
013	500	60	4	5	0	6	20
014	500	130	7	20	3	0	70
015	500	160	7	1	0	0	0

Abbreviation: ND, not determined.

for 1 patient showing a return of B cells 7 months after treatment.

Serum antibody pharmacokinetics. Serum antibody was detected in all patients immediately after the intravenous infusion. Serum levels following 100, 250, and 500 mg/m² infusions are shown in Fig 2. In the 9 patients receiving 100 mg/m² or greater, the mean half-life of the antibody was 4.4 days, with a range from 1.6 to 10.5 days. Antibody levels of greater than 10 μg/mL persisted in the serum of 6 of 9 patients for more than 14 days. In 3 of 6 patients treated with 250 mg/m² or 500 mg/m², antibody levels were detected 1 month after antibody therapy. In one case, the antibody half-life was 10.5 days and serum antibody, capable of binding to pretreatment tumor cells was present more than 1 month after treatment. One patient (patient no. 011) treated at the 250 mg/m² dose had a large tumor burden with splenomegaly and a short half-life of the chimeric anti-CD20 antibody (1.6 days).

Detection of host anti-IDEC-C2B8 immune response. Sera were analyzed at monthly intervals for the 3-month duration of the trial for evidence of an immune response. No quantifiable antibody responses were detected against the chimeric antibody, with a level of quantification of 5 μg/mL. Subsequent analysis of serum from 7 patients at various intervals to 1 year of follow-up were also negative.

Analysis of posttreatment tumor biopsy specimens. In patients receiving 100 mg/m² or greater doses of the chimeric antibody, excisional tumor biopsies were performed 2 weeks after antibody therapy. Tissue sections of these biopsies were examined for histopathology and cell suspensions examined using two-color flow cytometry to determine cellular composition and to look for evidence of the chimeric antibody bound to the tumor cells from the in vivo administration. Successful lymph node biopsies were obtained in 7 of these 9 patients. In 1 patient, a large node was resected; however, histologic examination showed necrosis without any viable

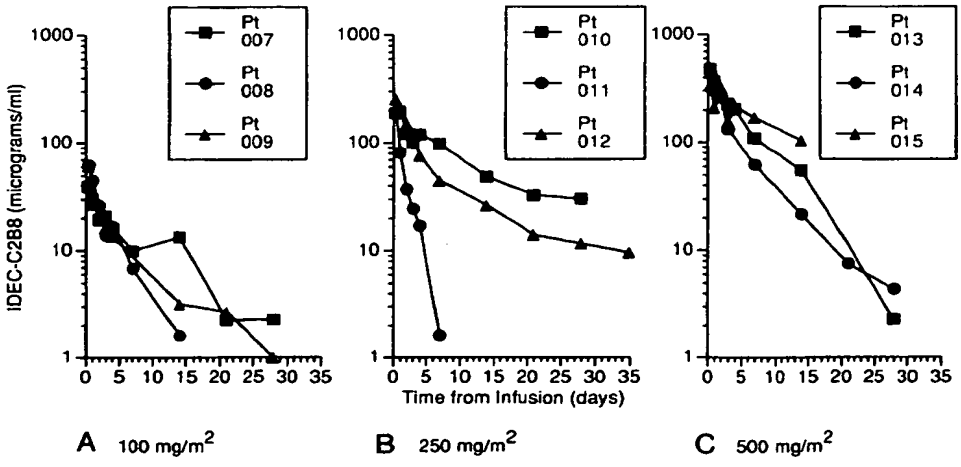

Fig 2. Pharmacokinetics of chimeric anti-CD20 antibody in patients receiving 100, 250, or 500 mg/m² infusions. Serum from 9 patients treated with a single infusion of the chimeric anti-CD20 antibody were analyzed for IDEC-C2B8 by ELISA. Results from each patient treated at 100 mg/m² (A), 250 mg/m² (B), and 500 mg/m² (C) are shown.

Table 4. Summary of Pretreatment and 2-Week Posttreatment Tumor Biopsies

Patient No.	Dose (mg/m²)	Date of Bx	Lymphoma Histology	Analysis by Flow Cytometry		
				% B (CD37)	% T (CD3)	C2B8 on Tumor Cells?*
007	100	10/9/90	Monocytoid B	79	21	—
		5/10/93	F&DMX	54	43	Yes (72)
008		3/24/92	FLC	53	43	—
		5/12/93	No tumor	—	—	—
009		9/16/92	FM	80	12	—
		5/17/93	Necrosis	—	—	—
010	250	6/4/87	FLC	63	26	—
		5/19/93	FM	51	44	Yes (30)
011		1/92	SLID	79	20	—
		5/26/93	SLID	83	16	No
012		5/11/93 fna	—	97	5	—
		6/1/93	FM	77	19	Yes (41)
013	500	10/28/92	FM	57	27	—
		6/1/93	FM	34	35	Yes (83)
014		5/11/93	FSC	85	12	—
		6/9/93	FSC	52	23	Yes (100)
015		6/7/93 fna	—	83	15	—
		6/22/93	FSC	60	40	Yes (93)

Abbreviations: fna, cells obtained by fine needle aspiration; FM, follicular mixed small and large cell; FLC, follicular large cell; FSC, follicular small cleaved cell; SLID, small lymphocytic intermediate differentiation (mantle cell); F&DMX, follicular and diffuse mixed small and large cell.

* Expressed as the percentage (shown in parentheses) of lymphoma cells in the biopsy specimen that stained positive for the expression of human IgG constant regions.

tumor. Flow cytometry also failed to detect any viable cells. A second patient had large bilateral inguinal lymph nodes present before therapy, but attempted surgical excisions in both inguinal regions after treatment failed to find any lymph nodes for biopsy. In the remaining 7 patients, lymph node material was available for analysis. Table 4 details the histologic findings present in the 2-week posttreatment tumor biopsies and compares the B- and T-cell content as determined by flow cytometry with tumor biopsies obtained before antibody therapy. In 3 patients, tumor biopsies were obtained immediately before treatment, and in the remaining patients comparisons were made to earlier cryopreserved cell suspensions obtained during the patient's course (from 8 months to 6 years earlier). Histologic examinations of the posttreatment samples remained diagnostic of lymphoma in the 7 patients. In 1 patient, the histologic appearance of the posttreatment tumor biopsy identified large numbers of hemosiderin-laden macrophages clogging the sinusoids of the lymph node. When the posttreatment samples were compared with the earlier biopsies, there appeared to be a decrease in the percentage of B cells (as determined by the expression of CD37 and CD19, both independent B-cell antigens not blocked by anti-CD20 antibodies) and a corresponding increase in the percentage of T cells seen in all but 1 patient. In addition, chimeric antibody was identified on the surface of the tumor cell population in the 2-week posttreatment biopsy in all but one case. In some cases, the bound antibody nearly completely saturated the available CD20 binding sites.

Effect on serum IgG, IgM, serum complement, and platelets. Sequential quantitative serum Ig levels were obtained pretreatment and monthly during the 3-month follow-up period. As shown in Fig 3, there was no significant change in the serum IgG (Fig 3A) or IgM (Fig 3B) levels over this period. IgA levels also were unchanged (data not shown). The platelet count was largely unaffected by the administration of antibody. One patient who started with a low pretreatment level developed moderate thrombocytopenia (Fig 3C). Two patients developed transient decreases in serum complement (C3) at 24 hours after therapy (Fig 3D).

Clinical antitumor effect. Despite the fact that this trial involved the administration of only a single infusion of antibody, tumor responses were observed. Partial tumor responses were documented in 2 patients and minor responses observed in 4 others. An example of a partial remission is shown in Fig 4. Patient no. 015, who had a follicular small cleaved cell lymphoma previously treated with ProMACE/MOPP, radiation therapy, and interferon was treated with 500 mg/m² of antibody. Computed tomography (CT) images of an abdominal mass pretherapy (Fig 4A and B) and 3 months posttherapy (Fig 4C and D) are shown. This tumor mass substantially decreased after treatment, and other nodes on CT scan and ultrasound examination as well as physical examination disappeared or significantly decreased in size. Disease progression was documented 8 months posttherapy, with recurrence of axillary adenopathy. A second patient treated with 100 mg/m² had a greater than 50% decrease in cervical adenopathy as well as resolution of splenomegaly (CT scan) lasting 9 months posttherapy. Patient no. 002, with follicular small cleaved cell lymphoma previously treated 10 years earlier with total nodal radiation and multiple courses of alkylating agents, was found on pretreatment evaluation to have circulating lymphoma cells and persistent thrombo-

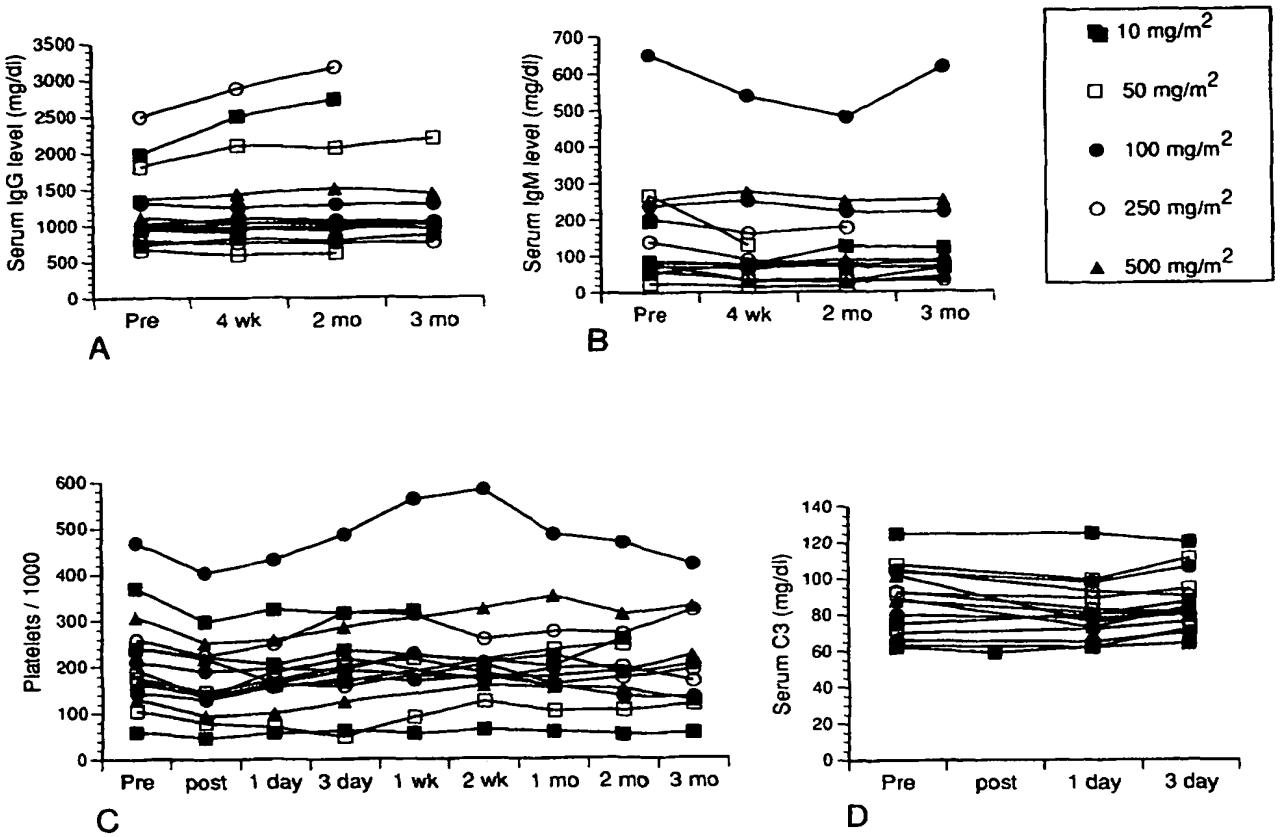

Fig 3. Effect of anti-CD20 antibody therapy on platelets, IgG, IgM, and serum complement levels. After therapy with a single infusion of chimeric anti-CD20 antibody patients were monitored for the effect on serum IgG (A), IgM (B), platelets (C), and serum complement-C3 (D).

cytopenia. He was treated with 10 mg/m^2 antibody. Throughout the 3 months of follow-up there was no significant change in disease measurement or thrombocytopenia. However, evaluation at 7 months showed a significant reduction in disease (lymph nodes and splenomegaly), resolution of thrombocytopenia, and disappearance of tumor cells from the peripheral blood lasting more than 1 year from therapy. Although no other lymphoma therapy had been administered, it is impossible to know whether the clinical improvement in this patient was spontaneous or was indeed related to the antibody treatment. Two patients had attempted excisional biopsies of known disease sites that showed necrosis in one instance and no tumor in a second case. Both patients had evidence of tumor regression with a mixed response and a minor response (25% to 50% decrease in measured lesions), the latter lasting 11 months. Shorter minor responses were also observed in 2 other patients. Additional mixed responses were observed in several patients. One patient with a history of low-grade lymphoma with transformation in bone to diffuse large-cell lymphoma had complete resolution of peripheral disease (last biopsy follicular mixed lymphoma), but progressed 2 months after therapy with relapsed large cell lymphoma again involving bone. He received local radiation therapy to the involved bone, but remains in remission of his peripheral disease.

DISCUSSION

In this phase I clinical trial, patients with relapsed NHL received a single infusion of chimeric anti-CD20 MoAb IDEC-C2B8 in doses ranging from 10 to 500 mg/m^2. All patients received the planned dose and no dose-limiting toxicities were identified. Symptoms were mild to moderate and easily manageable and more commonly observed in the 3 patients with higher numbers of CD20 antigen-bearing B cells (normal or malignant) present in the peripheral blood, suggesting that the destruction or removal of these cells during the early portions of the antibody infusion may contribute to the adverse events observed.

Analysis of antibody pharmacokinetics in patients receiving doses of 100 mg/m^2 or greater showed a mean serum half-life of 4.4 days, with a range from 1.7 to 10.5 days. However, it is difficult to establish the half-life of antibody in patients with widely different degrees of tumor burden receiving a single nonsaturating dose of antibody. It is likely that the true half-life will be longer once sufficient antibody is administered to saturate all tumor and normal CD20 antigenic sites. In the majority of patients receiving these doses, levels of greater than 10 μg/mL were present in the serum 2 weeks after therapy. Lower levels were identified in patients with extensive disease. Two-week postinfusion lymph

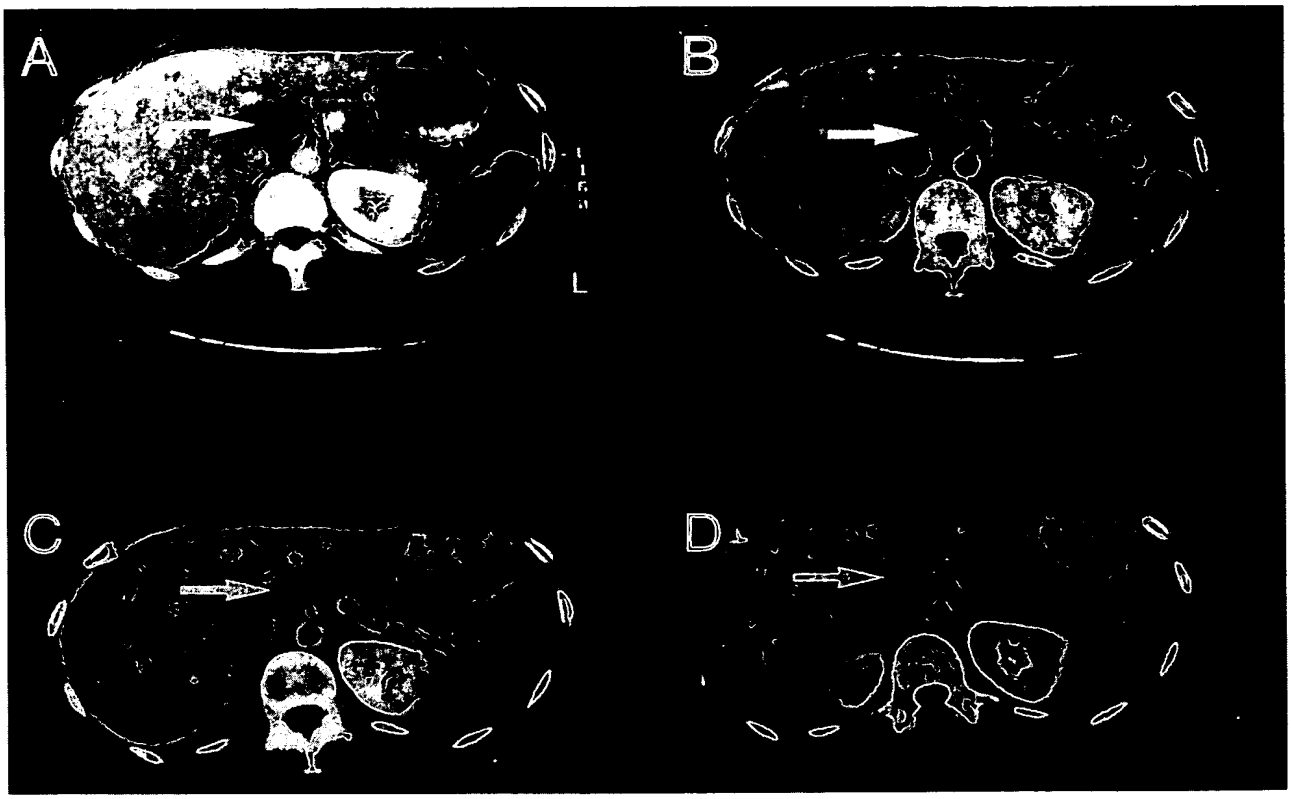

Fig 4. Example of antitumor effect. Patient no. 015 with relapsed follicular small cleaved cell lymphoma was treated with 500 mg/m² chimeric anti-CD20 antibody. CT images of an abdominal mass from two contiguous images are shown in (A) and (B). The corresponding images from a scan performed 3 months after antibody therapy are shown in (C) and (D), demonstrating partial regression of the tumor.

node biopsies were performed in the 9 patients receiving doses of greater than 100 mg/m². In 7 of the 9 biopsies, tumor was identified on pathologic examination. However, in 2 patients, tumor was not identified; in 1 case a large node that had become smaller after therapy was necrotic and in a second case inguinal lymph nodes present before therapy were not found after surgical exploration of left and right inguinal regions. In some of the biopsies, an increased infiltrate of macrophages was observed. Analysis of the posttreatment lymph nodes by flow cytometry identified tumor cells coated with nonsaturating amounts of the chimeric antibody in 6 of the 7 cases. The single exception was a patient with a large tumor burden, splenomegaly, and a short serum half-life of the administered antibody. In the majority of cases, a general decrease in the percentage of B cells and an increase in the percentage of T cells was observed when comparing pretreatment and posttreatment biopsies.

Treatment caused a selective elimination of the peripheral CD20-expressing B cells in all but 1 patient receiving doses of 100 mg/m² or greater. This was observed 24 to 72 hours after MoAb infusion. Over the 3-month follow-up phase, peripheral blood B cells slowly returned to base line in patients treated with the lower doses and partially in patients receiving the higher doses. There was no apparent effect on the serum IgG, IgM, or IgA levels through 3 months of follow-up. The CD20 antigen is not expressed on the early B-cell precursors or on the antibody-secreting plasma cell. The compartment of B cells/plasma cells is much larger than the few cells observed in the peripheral blood, and it is possible that they were not effected by the single dose of antibody. In addition, the long half-life of serum IgG may mean that late effects on serum IgG will be observed. There did not appear to be any increased incidence of opportunistic infections in this group of patients during the 3 months of follow-up. Depletion of normal B cells is common after high-dose chemotherapy and autologous bone marrow reconstitution that slowly resolves 3 to 6 months after transplant.[22,23] Depression of Ig levels can be treated by Ig transfusions.[24] It is possible that longer duration of antibody therapy achieving saturating levels may also cause a greater antitumor effect and a greater effect on the normal B cells and, ultimately, serum Ig.

The mechanism of the antibody-induced antitumor effect is not clear. Serum complement levels (C3) were slightly changed in only 2 patients during therapy. The chimeric antibody is capable of lysing target tumor cell lines in vitro by complement and antibody-dependent cell-mediated lysis.[21] In addition, direct effects of antibody binding to the

CD20 antigen, including growth inhibition, have been reported.[12] It is likely that a combination of these mechanisms is involved in the tumor regressions observed in these patients. Studies using murine anti-CD20 antibodies have also noted antitumor effects.[13] Two recent reports detailing the use of radiolabeled anti-CD20 antibodies describe impressive clinical activity with complete or partial remissions in the majority of patients.[14,15] Both also note tumor regressions associated with the imaging portion of the studies, suggesting clinical activity of the murine antibody.

The use of a chimeric naked antibody offers some advantages over similar trials using toxin-conjugated or radiolabeled antibodies against B-cell NHL. The antibody preparation is used directly for therapy, not requiring conjugation to drugs, toxins, or radiolabels, each of which requires extensive safety testing and may not be stable after formation of the active conjugate. Antibody modification may interfere with antigen binding. Radioiodinated antibodies are unstable, and undergo autolysis, and it is technically difficult to obtain consistent conjugates for large-scale clinical trials. In addition, significant hematologic toxicity is associated with the use of high-dose radiolabeled conjugates, making the application of this approach difficult in patients with impaired bone marrow function or significant involvement by lymphoma. In some studies, immunotoxin conjugates have been associated with significant toxicities.[25] In contrast, this chimeric anti-CD20 antibody is stable and has been engineered to lyse tumor cells through interaction with the patient's own immune system.

The modest tumor responses observed in this trial occurred after the administration of a single infusion of the chimeric antibody. Extension of these studies using multiple doses to achieve prolonged, tumor-saturating levels may lead to responses in patients with more extensive disease. Ultimately, extension of these studies to patients with minimal residual disease, using antibody alone or in combination with conventional therapies, may provide the greatest benefit. Based on these observations of safety and tumor responses to a single infusion of this chimeric anti-CD20 MoAb, a phase I/II trial using four weekly doses of antibody in patients with relapsed B-cell NHL has been initiated.

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SEQUENCE ALIGNMENT

COLON SPECIFIC PROTEIN (TOP LINE)

VS.

U.S. PATENT NO. 5,436,169 SEQ ID NO:7 (BOTTOM LINE)

Lipman-Pearson Protein Alignment
Ktuple: 2; Gap Penalty: 4; Gap Length Penalty: 12

Seq1(1>158) ColonSpecPro_SeqNo2.PRO	Seq2(1>174) USPat5436169_SeqNo7.PRO	Similarity Index	Gap Number	Gap Length	Consensus Length
(9>155)	(18>172)	29.8	5	10	156

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L:LLS :. ..   ::   R .C: G   . S:CY: F . ::W:DA:L.CQ.  :G :L.S:LS  E:S
LMLLSQVQGEEPQRELPSARIRCPKGSKAYGSHCYALFLSPKSWTDADLACQKRPSG-NLVSVLSGAEGS
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  ↓80        ↓90              ↓100       ↓110       ↓120       ↓130
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   ↑90        ↑100       ↑110       ↑120       ↑130       ↑140       ↑150
↓140       ↓150
TWSSNECNKRQHFLCK
 W.. :CN R .::CK
RWKDYNCNVRLPYVCK
   ↑160       ↑170
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O

Top

Orthologue

Orthologues are genes derived from a common ancestor through vertical descent. This is often stated as the same gene in different species. In contrast, paralogs are genes within the same genome that have evolved by duplication.

The hemoglobin genes are a good example. Two separate genes (proteins) make up the molecule hemoglobin (alpha and beta). The alpha and beta DNA sequences are very similar and it is believed that they arose from duplication of a single gene, followed by separate evolution in each of the sequences. Alpha and beta are considered paralogs. Alpha hemoglobins in different species are considered orthologs.

P

Top

Paralog

Paralogs are usually described as genes within the same genome that have evolved by duplication. See Ortholog.

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Biochemicals

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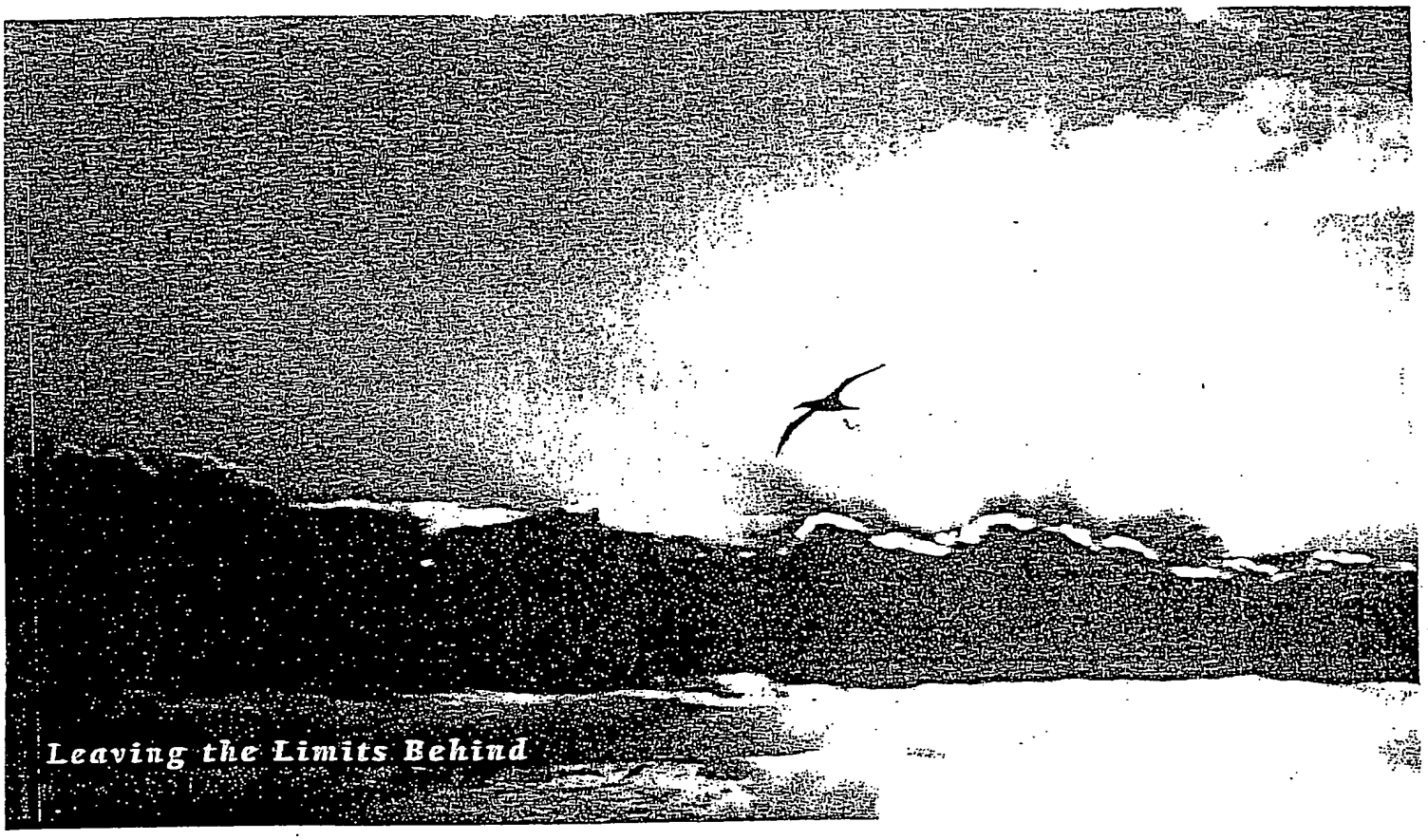

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Primary Antibodies

Antibodies to Human Leukocyte Antigens

CD	Antibody	Specificity and Notes	Species Specificity
CD1a	Anti-thymocyte (clone VIT6b) Cat. No. 1273 418	The antibody recognizes the heavy chain (49 kD) of the CD1a molecule and binds thymocytes, Langerhans cells in human skin, astrocytes in human brain, and endothelial cells in human spleen. The antibody may be used to detect thymocytes in suspensions of blood cells. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human
CD2	Anti-T pan (clone MT26) Cat. No. 1089 242	The antibody recognizes the CD2 antigen (46-50 kD) on most peripheral T lymphocytes (>70% of resting T cells and >80% of activated T cells) and thymocytes (80%) in humans. The antibody also binds T cells in frozen tissue sections of lymph and thymus. The CD2 antigen is the sheep erythrocyte receptor found on most human T lymphocytes. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human
CD3	Anti-T pan (clone 4B5) Cat. No. 1273 485	The antibody recognizes the CD3 complex (three proteins, 19–29 kD) on all peripheral T lymphocytes and mature thymocytes in humans. The antibody may be used to detect the total population of mature T lymphocytes or to study the mechanism of the CD3 complex. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human
CD9	Anti-leukemia associated antigen (p24) (clone BA-2) Cat. No. 1118 021	The antibody reacts with a monomeric 24 kD protein found on the surface of cells from about 75% of the cases of non-T, non-B acute lymphoblastic leukemia, and on normal immature lymphoid cells (lymphoid progenitor cells, which lack characteristics of either T or B cells); reacts weakly to cells from some T-acute lymphoblastic leukemias; binds 3% of peripheral blood lymphoid cells, and about 5-10% of bone-marrow mononuclear cells. BA-2 fixes rabbit complement; binds protein A. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at –20°C or colder.	human
CD10	Anti-common acute lymphoblastic leukemia antigen (CALLA) (clone 12A1) Cat. No. 1295 772	The antibody recognizes CALLA, a neutral endopeptidase (90 kD) found in cells from human pre-B cells, fibroblasts, granulocytes and most acute lymphoblastic leukemias (ALL). **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human
CD11a	Anti-lymphocyte function antigen (LFA) (clone 38) Cat. No. 1428 535	The antibody specifically recognizes the α-chain (CD11a) of the LFA-1 complex and immunoprecipitates a protein with a molecular weight of 175-180 kD from human monocyte lysates. The antibody reacts with nearly all peripheral blood leukocytes. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at –20°C or colder. Avoid repeated freezing and thawing.	human
NEW CD11b	Anti-macrophage (clone 44) Cat. No. 1441 256	The antibody recognizes the complement receptor type 3 (CD11b), a plasma membrane glycoprotein on human monocytes. The antibody inhibits the aggregation of activated neutrophils by greater than 50%. The antibody immunoprecipitates a protein from the surface of neutrophils with an apparent molecular weight of 165 kD. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at –20°C or colder. Avoid repeated freezing and thawing.	human
CD11b/ CD18	Anti-Mac-1 (macrophage associated antigen) (clone M1/70) Cat. No. 1118 129	The antibody specifically reacts with native mouse and human Mac-1 (complement receptor type 3; Ly-40) antigen and precipitates two chains, 170 kD (CD11b) and 95 kD (CD18). **Stability:** Stable at –20°C. Avoid repeated freezing and thawing.	human, moust
NEW CD11c	Anti-lymphocyte function antigen-1α (LFA-1α) (clone BL-4H4) Cat. No. 1484 311	The antibody specifically recognizes the α-chain (CD11a) of the LFA-1 complex and immunoprecipitates a protein with a molecular weight of 150 kD from human monocyte lysates. The antibody reacts with nearly all peripheral blood leukocytes. **Stability:** Stable for 6 months at +4°C. Alternatively, store in aliquots at –20°C. Avoid repeated freezing and thawing.	human
CD14	Anti-myeloid cells (clone AML2-23) Cat. No. 1118 188	The antibody is cytotoxic in the presence of complement. The antibody binds an antigen (50 kD) found on acute myeloid leukemias and some acute promyelocytic leukemias. It also binds normal monocytes, macrophages and neutrophils. **Reference:** Ball, E.D. *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5374. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at –20°C or colder. Avoid repeated freezing and thawing.	human
CD15	Anti-granulocyte (clone 4D1) Cat. No. 1295 845	The antibody recognizes lacto-N-fucopentose III, a carbohydrate antigen found on human neutrophil granulocytes. The antigen is also found on Reed-Sternberg and Hodgkin's (lymphoma) cells. The antibody also binds some secretory gland tissue and epithelium from esophagus and cervix. The antibody reacts with the CD15 antigen (expressed late in the development of granulocytes) on cells in suspension and in frozen tissue. The antibody may be used to detect mature granulocytes in suspensions of blood cells. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human

B = Southern/northern/dot blots
C = Cryosections
CD = Cluster of Differentiation
E = ELISA
FC = Flow cytometry
IC = Immunocytochemistry
IHC = Immunohistochemistry
IP = Immunoprecipitation
N = Neutralizing
P = Paraffin sections
S = Histological sections
W = Western (proteins)

Note: Listed applications are tested by Boehringer Mannheim. Any application not listed does not necessarily indicate that the antibody does not work in that application.